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Astrocytic CB1R-mediated calcium signalling: impact in glutamate transport and interaction with adenosine receptors

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Mestrado em Biologia Molecular e Genética

Dissertação orientada por:
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2018

“The Most Exciting Phrase in Science Is Not ‘Eureka!’ But ‘That’s funny …’”

Isaac Asimov

Abstract

Astrocytes are the major glial cell type in the central nervous system (CNS) being responsible for many actions in the CNS. Astrocytes express several types of G-protein coupled receptors, namely the cannabinoid type one receptors (CB1R) that are activated by endocannabinoids released from neurons. Usually these receptors are coupled to $G_{i/o}$ proteins although there are reports of $G_{q/11}$ coupling. In astrocytes, CB1R activation leads to phospholipase C (PLC)-dependent Ca^{2+} mobilization from internal cellular stores, which will then have key roles in brain function. Astrocytes also express adenosine A_{2A} receptors ($A_{2A}R$) that, when activated by adenosine, have several modulatory effects, such as the control of glutamate transport. Thus, both type of receptors, CB1R and $A_{2A}R$, are of pivotal importance in modulation of neuronal excitability. Interaction between CB1R and $A_{2A}R$ have been well studied in neurons, nevertheless nothing is known concerning this crosstalk in astrocytes. From the several roles of astrocytes in the CNS, the glutamate uptake mediated by specific glutamate transporters is one of the most relevant functions, since this function prevents excitotoxicity due to glutamate accumulation in the synaptic cleft. To achieve the suitable glutamate transport from the extracellular space, astrocytes express mainly the glutamate transporters GLAST and GLT-1.

Having in mind the lack of information regarding the effect of CB1R in astrocytes, the main aim of this work was to study the role of CB1R activation-mediated calcium signaling upon GLAST transporter in rat primary astrocytic cultures. This study was designed to better understand the role of CB1R activation on astrocytic calcium signalling and to study its relevance upon glutamate transport. Furthermore, this work aimed to study the crosstalk between CB1R and $A_{2A}R$ upon calcium signaling and glutamate uptake.

Primary astrocytic cultures from cortex were prepared from Sprague Dawley pups (0-2 days old). Calcium signaling and [^{3}H]-glutamate uptake experiments were performed with 18-23 DIC astrocytes. Calcium signaling experiments were performed using FURA-2AM dye and specific transport mediated by GLAST was achieved by the difference of total glutamate transport and transport in presence of UCPH-101, a specific blocker of this transporter.

In primary cultures of astrocytes it was observed that CB1R activation with ACEA led to calcium transients, whose amplitude and frequency was dose-dependent for the two tested ACEA concentrations (0.5 and 1.0 µM). The occurrence of these transients was abolished in the presence of antagonists of CB1R, AM251 (1 µM), of PLC, U73122 (3 µM) and a chelator of intracellular calcium stores, CPA (10 µM). Low extracellular calcium concentration decreased both frequency and amplitude of the transients while inhibition of $G_{i/o}$ protein, with PTx (5µg/ml), decreased significantly frequency with no significant changes in amplitude. By performing glutamate uptake assays, it was observed that GLAST was the main glutamate transporter in primary astrocytic cultures. CB1R activation increased significantly the V_{max} kinetic constant of the GLAST transporter. This potentiation involved PLC and calcium signalling, but was PKC independent. When evaluating the crosstalk between CB1R and $A_{2A}R$, it was observed that activation of $A_{2A}R$ decreased both frequency and amplitude of CB1R-mediated calcium transients. On the other hand, when the $A_{2A}R$ was inhibited, CB1R-mediated transients frequency was higher when compared to ACEA incubation alone. When adenosine A_1 receptor was previously inhibited, the CB1R-elicited transients 'frequency seemed to be decreased.

In conclusion, CB1R activation triggers calcium transients in astrocytes by PLC activation, in a dose-dependent manner. This calcium signaling then enhances glutamate transport probably by increasing the number of functional GLAST transporters in the plasma membrane. In parallel, adenosine $A_{2A}R$ activation decreases CB1R activity. This highly suggests that, in a synapse context, endocannabinoids

released by post-synaptic neurons will activate astrocytic CB1Rs, increasing glutamate transport and contributing to clear up excessive glutamate at the synaptic cleft, and that this effect is possibly controlled by adenosine A_{2A} receptors.

Keywords: Astrocytes, CB1R, Adenosine A_{2A} receptors, Calcium signalling, Glutamate uptake

Resumo

Os astrócitos são células multifuncionais que coexistem com os neurónios no sistema nervoso central, cuja morfologia pode variar conforme localização e/ou função. Na sinapse, interagem direta e indiretamente com os terminais pré-sinápticos, pós-sinápticos, e astrócitos vizinhos, uma vez que possuem recetores específicos para neurotransmissores, sendo capazes de libertar gliotransmissores que, por sua vez, se ligam a recetores presentes nos terminais nervosos. Esta comunicação bidirecional entre astrócitos e neurónios leva então ao conceito de sinapse tripartida.

Além da gliotransmissão, os astrócitos também são responsáveis pela maioria da captação de glutamato nas sinapses. O glutamato é o principal neurotransmissor excitatório com um papel crucial na sinalização neuronal. Após ter cumprido a sua função como neurotransmissor, é necessário a saída rápida deste da fenda sináptica de modo a impedir excitotoxicidade. Para tal, astrócitos e neurónios expressam transportadores de glutamato. A atividade destes transportadores é controlada de forma fina por vários componentes intra e extracelulares como fatores de crescimento, neuromoduladores, sinalização de cálcio, etc..

Atualmente, cannabis é a droga ilícita mais consumida nos países desenvolvidos. A planta *cannabis sativa* possui centenas de substâncias psicoativas, como o THC, que se ligam a recetores específicos no cérebro e periferia que, iniciando uma cascada de eventos, levam aos efeitos psicoativos da cannabis, já conhecidos. Hoje em dia sabe-se que os mamíferos são capazes de produzir e degradar endocanabinóides que também se ligam a estes recetores funcionando como neurotransmissores retrógrados. O sistema endocanabinóide passou a ser conhecido por um sistema regulador homeostático com uma sinalização intra e intercelular. Os recetores de canabinóides mais estudados são os recetores de cannabinóides tipo 1 (CB1R) e recetores de cannabinóides tipo 2 (CB2R). O CB1R tem uma expressão mais dominante no sistema nervoso central (SNC) enquanto o recetor CB2 é mais expresso no sistema imunitário.

Durante a transmissão sináptica, os terminais pré-sinápticos libertam neurotransmissores na fenda sináptica que, por sua vez, se vão ligar aos recetores expressos nos terminais pós-sinápticos. Uma vez ligados, dependendo do tipo de neurónio/célula, os neurotransmissores podem conduzir a uma variedade de respostas, como inibição, excitação e/ou síntese de segundos mensageiros. Por vezes, os terminais pós-sinápticos respondem ao estímulo através da síntese de endocanabinóides. Estes, por sua vez, são libertados na fenda sináptica ligando-se e ativando os recetores canabinóides. No cérebro, a atividade desses recetores pode afetar múltiplas funções biológicas, como perceção da dor, digestão, aprendizagem, memória, ansiedade e funções cognitivas. Isso é feito através da regulação da transmissão sináptica e da plasticidade, como a inibição da libertação do transmissor, o controlo da excitabilidade neuronal e a regulação da plasticidade sináptica de curto e longo prazo.

O CB1R é um recetor acoplado à proteína G (GPCR) do sistema canabinóide com sete domínios transmembranares. A expressão deste não é uniforme ao longo do cérebro, sendo mais expresso no cerebelo, gânglios basais e córtex. Todavia, a baixa expressão do recetor não significa necessariamente que a sua função não é relevante. Nos neurónios, os CB1Rs são geralmente acoplados a $G_{i/o}$ e, em alguns casos, a G_s, enquanto que nos astrócitos há evidências deste recetor estar acoplado a $G_{q/11}$. Nestas células, sabe-se que a ativação deste recetor leva à mobilização de cálcio das reservas intracelulares através da atividade de fosfolipase C (PLC).

Semelhante ao CB1R, os recetores de adenosina são um grupo de proteínas GPCR que são ativadas por ligação com a adenosina. Até à data, quatro subtipos de recetores de adenosina foram descobertos e clonados: A_1, A_{2A}, A_{2B} e A_3. Os recetores A_1 e A_3 de adenosina são inibitórios sendo acoplados a $G_{i/o}$ inibindo a acumulação de cAMP e a atividade de proteína quinase A (PKA), enquanto que os recetores

A_{2A} e A_{2B} são excitatórios, acoplados a proteínas Gs cuja ativação leva ao aumento de cAMP e ativação de PKA. No SNC, a adenosina é considerada um neuromodulador potente e os recetores A1 e A2A de adenosina estão associados a funções cerebrais críticas, como a libertação e captação de neurotransmissores e plasticidade sináptica. Nos astrócitos, a atividade dos recetores A2A de adenosina está correlacionada com a captação diminuída de aspartato e a regulação da memória, enquanto que os recetores A1 estão correlacionados com a ativação da PLC, sono e fatores de crescimento.

O objetivo deste estudo foi entender melhor a sinalização de cálcio mediada pelo CB1R e averiguar a sua relevância na atividade de transportadores de glutamato em astrócitos, assim como estudar a interação entre os recetores de adenosina e o CB1R em astrócitos. Para este fim, foram realizados ensaios de imunocitoquímica, imagiologia de cálcio e captação de glutamato radioativo em culturas primárias de astrócitos.

As culturas primárias de astrócitos foram preparadas do córtex cerebral de Sprague Dawley com 0-2 dias, sendo usadas células com 3 semanas em cultura. Nos ensaios de imagiologia de cálcio, foi observada a evolução do cálcio intracelular ao longo do tempo, incubando a cultura de astrócitos com uma sonda FURA-2AM. Nos ensaios de captação de glutamato, foi vista a quantidade de glutamato radioativo incorporada pelos astrócitos. A captação mediada pelo transportador GLAST ou GLT-1 é dada pela diferença entre o transporte total (na ausência de qualquer antagonista) e o transporte obtido quando um destes transportadores é bloqueado.

Em imunocitoquímica, a expressão do CB1R, assim como dos principais transportadores de glutamato GLT-1 e GLAST, foi encontrada em culturas primárias de astrócitos. A expressão dos transportadores GLAST e GLT-1 não foi uniforme. O GLT-1 apresentou uma expressão mais alta na área ao redor do núcleo enquanto que o GLAST aparentou ter uma expressão mais alta na membrana e no espaço intracelular.

Em imagiologia de cálcio, a presença do agonista seletivo do CB1R induziu transientes de cálcio, sendo que a amplitude e a frequência destes era dependente da concentração de agonista. Na presença de um antagonista seletivo do CB1R, o efeito foi abolido, levando à conclusão que a sinalização de cálcio induzida por ACEA é mediada pela ativação do CB1R. Esta sinalização de cálcio foi abolida na presença de um depletor de reservas de cálcio intracelulares enquanto que, em condição de baixa concentração de cálcio extracelular, a amplitude e frequência diminuíram. Na presença de um antagonista de PLC (U73122), o efeito também foi abolido. Quando a proteína $G_{i/o}$ foi inibida na presença de um antagonista especifico, PTx, a frequência destes diminuía sem alterações na amplitude, sugerindo fortemente algum tipo de participação por parte de um recetor que não o CB1R acoplado a $G_{i/o}$ na sinalização de cálcio. Quando o crosstalk entre CB1R e A_{2A}R foi avaliado, observou-se que a pré-ativação de A_{2A}R diminuiu a frequência e a amplitude dos transientes de cálcio mediados por CB1R. Por outro lado, quando o A_{2A}R foi inibido, a frequência de transientes mediada por CB1R aumentou quando comparada à incubação de ACEA isolado. Quando o recetor de adenosina A_1 foi previamente inibido, a frequência de transientes induzida pelo CB1R pareceu diminuir.

Em ensaios de *uptake* de glutamato, a presença de ACEA aumentou a captação de glutamato em astrócitos, através de um aumento da constante cinética V_{max} na atividade do transportador GLAST sem diferença em K_m. Na presença do antagonista do CB1R, este efeito foi abolido, assim como na presença de antagonista de PLC. Já na presença de um antagonista de PKC, uma proteína abaixo na sinalização da via de sinalização de PLC, observou-se uma potenciação do transporte, o que indica que esta proteína é provavelmente não participativa na via. Para saber se a sinalização de cálcio e potenciação do transporte de glutamato mediadas pela ativação do CB1R são efeitos paralelos ou em série, ativou-se o CB1R na presença de um quelante de cálcio. Nesta condição, não se observou potenciação de transporte

de glutamato, o que leva a crer que a sinalização de cálcio iniciada pela ativação de CB1R leva à potenciação da atividade do transportador GLAST.

Concluindo, em astrócitos, a ativação do CB1R induz transientes de cálcio intracelulares cuja amplitude e frequência têm um comportamento dose-resposta. A ativação de PLC e as reservas de cálcio intracelulares são essenciais para a ocorrência destes transientes. Isto sugere fortemente o acoplamento de $G_{q/11}$ ao CB1R, que está de acordo com estudos anteriores. Por sua vez, estes transientes aumentam o transporte de glutamato através do aumento da atividade do transportador GLAST, numa via independente de PKC. A inibição do recetor de adenosina A_{2A} aumenta a sinalização de cálcio mediada pelo CB1R, implicando uma interação direta entre estes dois recetores.

Palavras-chave: Astrócitos, CB1R, recetor de adenosina, sinalização de cálcio, captação de glutamato

Agradecimentos

Agradeço primeiramente à professora Ana Sebastião pela oportunidade de desenvolver a minha dissertação e de me permitir evoluir como investigadora no seu laboratório. Ao Instituto de Medicina Molecular que me recebeu tão bem e por facultar um ambiente excelente que nos leva a querer ser sempre melhores.

À minha orientadora, Sandra Vaz, por não só me guiar ao longo desta dissertação, como também pelos conselhos, conversas e apoio ao longo deste ano. Por não ter sido só uma orientadora mas também uma amiga. Evoluí imenso como investigadora neste curto período de tempo e em grande parte deve-se a ti. Obrigada por teres sempre acreditado em mim.

Agradeço também ao meu co-orientador Professor Doutor Carlos Farinha por toda a disponibilidade e ajuda que deu ao longo da minha dissertação.

A todos os meus colegas de laboratório, em especial às astrócitas (e astrócitx) Ana, Joana, Nádia, Irene e Tatiana. Obrigada por sempre me terem ajudado quando precisei, pelas conversas e conselhos. A vossa ajuda foi crucial para este trabalho. Às beer hours e aos cafés pouco bebíveis e a muitas outras aventuras. O que aconteceu na FENS party fica na FENS party.

Aos amigos que me acompanharam nesta jornada ao longo destes dois anos. À Patrícia, Angelykah, Ana, Diana, Guilherme e Rita. Desesperar em conjunto sempre é melhor que desesperar sozinha.

À minha primeira "aluna" Oksana. Espero que te tenha ensinado algo e que tenhas gostado das estrelas que são os astrócitos. Se por acaso achas que ainda tenho de melhorar a ensinar, lembra-te que ainda só tenho n=1.

À Vitória e ao Manuel Maria. Não têm noção do impacto que tiveram na minha vida profissional (que ainda agora começou). Muito obrigada pelas grandes conversas e conselhos.

Aos meus avós que sempre me ajudaram como puderam, mesmo quando eu disse que não era necessário. Aos ovos estrelados com salsichas e batata frita.

À minha família por me aturar há tanto tempo. Eu sei que não é fácil! À Bela que sempre mostrou imenso entusiasmo ao longo deste ano. Às minhas irmãs Ana e Sara, desculpem todas as conversas que tive sobre a minha dissertação, se bem que ao menos podiam fingir que estavam interessadas. E finalmente, ao meu pai por me ensinar a exigir o máximo de mim própria e à minha mãe por me ter ensinado a ser melhor pessoa. Aos dois por me terem deixado descobrir o que queria e por me terem sempre ajudado nesse sentido.

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Acronym List

2-AG	2-arachidonoyl glycerol
A_1R	Adenosine type one receptor
$A_{2A}R$	Adenosine type two receptor
AC	Adenyl cyclase
ACEA	Arachidonyl-2'-chloroethylamide
ADA	Adenosine deaminase
AEA	Anandamide
AM 251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
ALS	Amyotrophic Lateral Sclerosis
BAPTA-AM	Acetoxymethyl bis(O-aminophenoxy)ethane-N,N,N',N'-tetraacetate
Ca^{2+}	Calcium ion
CAMKII	Calcium-Calmodulin-Dependent Protein Kinase II
cAMP	Cyclic adenosine monophosphate
CB1R	Cannabinoid type one receptor
CB2R	Cannabinoid type two receptor
CPA	α-Cyclopiazonic Acid
DAG	Diacylglycerol
DAGL	Diacylglycerol Lipase
DIC	Days in culture
EAAT1	Excitatory amino acid transporter one
EAAT2	Excitatory amino acid transporter two
eCB	Endocannabinoid
EGTA	Ethyleneglycol bis(β-aminoethylester)-N,N,N',N'-tetraacetate
ER	Endoplasmic reticulum
FAAH	Fatty acid amide hydrolase
FDA	Food and Drug Administration
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter
GPCR	G-protein coupled receptor

GPR55	G-protein-coupled receptor 55
GS	Glutamine synthetase
IP3	1,4,5-inosotol triphosphate
IP3R	1,4,5-inosotol triphosphate receptor
K^+	Potassium ion
K_m	Affinity
LTD	Long term depression
V_{max}	Maximum velocity of transport
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAPE	N-arachidonoyl phosphatidyl ethanol
NCX	Sodium-calcium exchanger
NMDAR	N-methyl-D-aspartate receptors
NO	Nitric oxide
PLC	Phospholipase C
U73122	1-6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione
PD	Parkinson's disease
PDL	Poly-d-lysine
PGE	Prostaglandin E
PIP2	Phosphatidylinositol 4,5 bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PTx	Pertussis toxin
ROI	Region of interest
RT	Room temperature
THC	(-) Δ9-tetrahydrocannabinol
TRPC	Transient receptor potential cation channel

1. Introduction

1.1. Cannabinoids

1.1.1. Cannabis and the Endocannabinoid system: a brief history

The drug cannabis, also known as marijuana, comes from the plant *cannabis sativa* and is mostly used for recreational or medical purposes although its consumption is sometimes linked to religious or spiritual purposes. Its first dated consumption comes from 2737 BC by Chinese emperor Shen Nung when it was described as a psychoactive agent. Currently, cannabis is the most consumed illicit drug in many developed societies. According to European Drug Report of the European Monitoring Centre for Drugs and Drug Addiction[1], in 2017, 23.5 million Europeans aged between 15 and 64 years consumed cannabis, among which 17.1 million young adults aged 15-34.

Fifty years ago, the reason why cannabis affected humans the way it did was unknown. It all started in 1964, when Raphael Mechoulam[2] and his group elucidated the structure of one of the main psychoactive compounds of *cannabis sativa*, (-) Δ9-tetrahydrocannabinol (THC). Due to lipophilic nature of cannabinoids its study showed to be more difficult and complex than other molecules. More than two decades of research and technical development were needed before the first THC-binding site of cannabinoid receptor type one (CB1R) was identified in 1993[3].

In 1992, Mechoulam's research group identified the endocannabinoid (eCB) anandamide. These discoveries lead to the understanding that not only mammalian organisms had specific receptors for the psychoactive compound of cannabis, but these same organisms were also able to synthetize similar lipophilic ligands that would activate them. The final conclusion of these studies was that mammalians have an eCB system, able to modulate a variety of physiological processes such as appetite[4], immune function[5], thermoregulation[6], and sleep[7].

Since the discovery of THC, the use of cannabis and cannabis-based pharmacology has been debated. Health and psychological effects upon cannabis consumption have yet to be fully discovered and/or understood, originating a big debate on the subject and if its consumption can be a benefit or a hazard to human health. This duality is more accentuated since cannabis is classified as a Schedule drug 1, a substance defined as a drug not currently accepted for medical use and with a high potential for abuse, making difficult to set up human trials . Both benefits and adverse effects have been reported upon acute and chronic cannabis consumption. Consumption of cannabis seems to increase future consumption of stronger drugs like heroin, both in humans and in rats[8,9]. The consumption of cannabis also appears to have a negative effect on pregnancy leading to lower baby weight and increased chance of premature birth[10]. On the other hand, cannabis consumption has been reported to ameliorate the symptoms of some neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS)[11] and epilepsy. Consumption of cannabis has also been shown to be an effective therapeutic treatment for chronic pain[12], nausea, and vomiting in the context of chemotherapy[13]. It also appears to be a potential candidate in Parkinson's disease (PD) treatment, preventing neurodegeneration by maintaining homeostasis of mitochondria[14] and, in humans, it was proven to improve motor and non-motor symptoms in PD[15]. In the context of psychiatric disorders, THC appears to magnify the hazards of some psychiatric diseases like schizophrenia disorder[16]. However, other cannabinoid found in cannabis, such as cannabidiol, are able to ameliorate schizophrenia disorder[17]. This shows that cannabinoid receptors can have different effects when activated by different types of cannabinoids.

Despite all of the debate, some cannabis-based drugs are Food and Drug Administration (FDA) approved and are currently in the market like Sativex, which is used for pain and multiple sclerosis[18]

since 2011. More recently, another cannabis-based drug, Epidiolex for seizures[19], is in course of being approved by FDA.

1.1.2. Cannabinoid System

Due to their lipophilic nature, cannabinoids are only able to signal in a paracrine or autocrine way, instead of a systemic way. Since the isolation of the main active compound of cannabis, much has been unfolded about the cannabinoid system present in mammalians. It consists of cannabinoid receptors such as cannabinoid type one receptor (CB1R) and cannabinoid type two receptor (CB2R), endogenous transmitters (eCBs) such as 2-arachidonoyl glycerol (2-AG) and anandamide (AEA), and the enzymatic machinery responsible for the eCBs production and degradation such as the fatty acid amide hydrolase (FAAH) and Diacylglycerol Lipase (DAGL). (For review see [20])

ECBs precursors are present in lipid cell membranes[21] which is one of the reasons why eCBs are widespread throughout the organism. Both 2-AG and AEA contain arachidonic acid but possess different pathways of synthesis and degradation with distinct enzymes. In general, AEA is produced by N-arachidonoyl phosphatidyl ethanol (NAPE) and 2-AG by enzymes such as diacylglycerol lipase (DAGL) and Phospholipase C (PLC). 2-AG binds to both CB1R and CB2R while AEA is more specific to CB1R (Figure 1.1).

In the synapse, eCBs are a type of retrograde neurotransmitters, that work as feedback for neurotransmission release. During neurotransmission, presynaptic neurons release neurotransmitters into the synaptic cleft which then bind to specific post synaptic neuronal receptors. Once bound to the specific receptors, depending on the type of neuron/cell, it will lead to a variety of responses such as inhibition, excitation, and/or synthesis of second messengers. Some cells such as post-synaptic neurons can also respond to the stimulus through the synthesis of eCBs. Much is yet to discovered about the synthesis process of eCBs although it is known that an intracellular calcium concentration ($[Ca^{2+}]_i$) elevation is required[22]. Once synthetized, eCBs are released into the synaptic cleft by a mechanism yet to be understood[23]. These eCBs then bind to cannabinoid receptors leading to their activation (Figure 1.1). As previously mentioned, in the brain, the activity of these receptors can affect multiple biological functions such as pain perception[12], food intake[24], learning and memory[25], and anxiety[26].

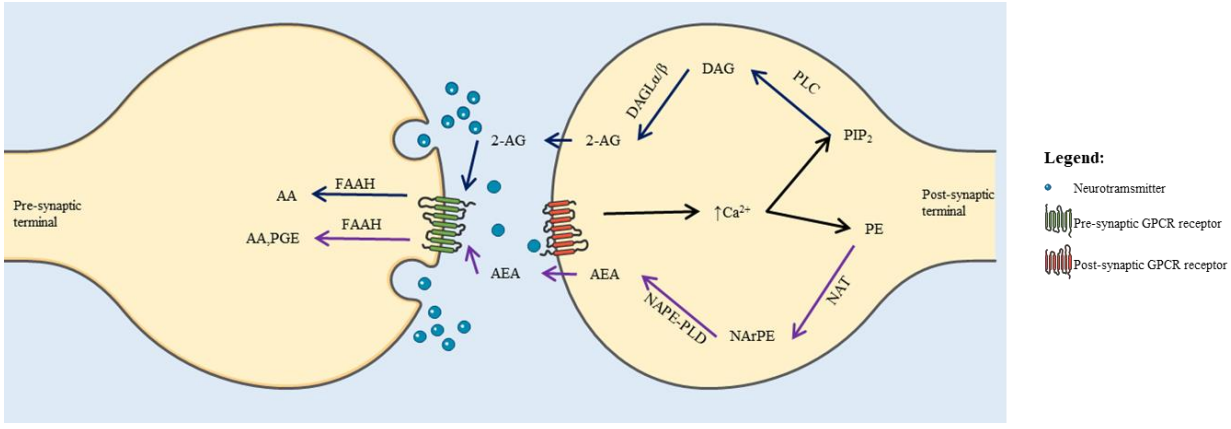

Figure 1.1 Endocannabinoids Pathways.
Main synthetic and degradative pathway for anandamide (in blue) and A-AG (in violet). Only the main pathways are shown. Abbreviations: AA, arachidonic acid; DAG, diacylglycerol; DAGL, diacylglycerol lipase ; FAAH, fatty acid aminohydrolase; NAPE-PLD, N-arachidonoyl phosphatidyl ethanol-preferring phospholipase D; PIP2, phosphatidyl inositol bis-phosphate; PLC, phospholipase C; PE, phosphatidyethanolamine; NArPE, N-arachidonoyl-PE, NAT, N-acyltransferase; AEA, anadamide.

Both receptors, CB1R and CB2R, are G-protein coupled receptors (GPCR) containing seven transmembrane domains. Their ability to regulate intercellular components comes through the activation of inhibitory $G_{i/o\alpha}$[27] and or G_s proteins[28], although there is a report of CB1R coupled with $G_{q/11}$[29], whose activation leads to $[Ca^{2+}]_i$ changes. Some reports have been stated that G-protein-coupled receptor 55 (GPR55) is cannabinoid-sensitive, but its classification as a cannabinoid receptor is controversial, due to the fact that in GPR55 knockout mice, the administration of atypical cannabinoids in order to activate this receptor showed no difference in blood pressure compared to wild types[30].

1.1.3. CB1 receptors in the brain

As it was mentioned before, CB1R is a GPCR with seven transmembrane domains, found widespread throughout the brain. CB1R is the most expressed GPCR protein in the brain, but its expression is not uniform throughout the brain, being more permanent in the cerebellum, ganglia basal, and cortex[31]. It is also expressed in the peripheral nervous system (PNS) mainly in sympathetic nerve terminals[32] being involved in pain, energy, metabolism, cardiovascular and reproductive functions, inflammation, glaucoma, cancer, and liver and musculoskeletal disorders[33]. Low expression of CB1R does not necessarily mean its function is not relevant. In glutamatergic neurons, activation of CB1Rs decreases excitatory postsynaptic currents and has a decreased amount of expression of CB1R, when compared to GABAergic neurons[34]. This is done through the regulation of synaptic transmission and plasticity such as inhibition of transmitter release, control of neuronal excitability, and regulation of short- and long-term synaptic plasticity[35]. CB1R activation inhibits glutamate and GABA release from pre-synaptic terminals modulating synaptic transmission[36,37].

In neurons, CB1Rs are usually coupled with $G_{i/o}$[2], whose activation inhibits cyclic adenosine monophosphate (cAMP) production and consequent protein kinase A (PKA) activation. There are also reports of G_s coupling where the exact opposite occurs[38]. As for astrocytes, there is evidence of $G_{q/11}$ coupling[29,39], although this type of coupling has never been proven in any type of brain cell through immunoprecipitation (Figure 1.2). In the hippocampus, astrocytic CB1R activation increases $[Ca^{2+}]_i$, triggering glutamate release that binds to pre-synaptic N-methyl-D-aspartate receptors (NMDARs) regulating spike timing-dependent depression[40]. It is important to keep in mind that these receptors do not seem to have many "intrinsic" properties, meaning the outcome of their activation can vary among brain regions. This probably comes from the ability of CB1R to couple to different GPCR and to form heterodimers/oligomers leading to different responses among tissue. For example, in neurons these receptors can form heterodimers with D2 dopamine receptors[28].

To better understand the mechanism behind CB1R signalling, many research groups use CB1R knockout mice. At first, these animals appear healthy but they have some significant differences from wild-type animals such as increased mortality rate and hypoactivity[41], defected adult neurogenesis[42], and are resistant to diet forced obesity[43].

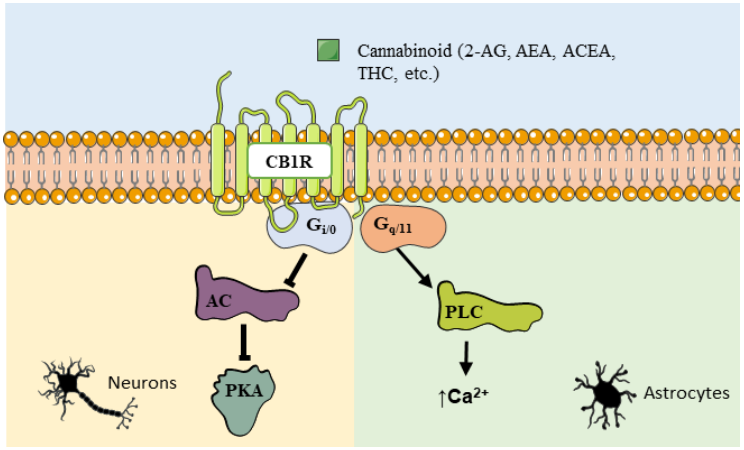

Figure 1.2- CB1Rs signalling pathways in neurons and astrocytes.
In neurons, CB1R is usually coupled to inhibitory $G_{i/o}$ proteins. Neuronal CB1R activation has been associated with adenyl cyclase (AC) inhibition, decreasing PKA activity. In astrocytes, there have been reports of CB1Rs coupled to $G_{q/11}$ although there are no immunoprecipitation reports. Astrocytic CB1R activation triggers intracellular calcium elevations eliciting neurotransmitter release.

1.2. Astrocytes

Glial cells are a type of non-neural cells located in the CNS and PNS that exist in the same number as neurons in the CNS[44] and the major distinction between them is that glial cells do not fire action potentials. Glial cells include astrocytes, oligodendrocytes, and microglia and together they maintain homeostasis in the nervous system.

Oligodendrocytes cover neuronal axons with their specific cell membrane known as the myelin sheath. This provides insulation in the axon leading to a more efficient propagation of signal[45]. Microglia are responsible for the immune defenses in the CNS and mediate inflammatory responses[46].

Astrocytes are restricted to the brain and spinal cord and their function and morphology can depend on their location. In the beginning, these cells were thought to be supportive cells for neurons, but today it is known that astrocytes are crucial for the maintenance of the CNS. Astrocyte function is very broad, ranging from brain homeostasis to the maintenance of neuronal metabolism. Between them, they have gap junctions making communication between these cells quite fast. Astrocytes also have a close interplay with blood vessels, performing uptake of water and glucose from vessels and releasing prostaglandin E (PGE) and nitric oxide (NO) to make vessels dilate and AA to contract them[47]. This type of glial cell can also control the supply of neurotransmitter left in synaptic cleft mainly through the uptake of neurotransmitters by specific transporters. For example, astrocytic glutamate transporters GLAST and GLT-1 have been found responsible for about 90% of glutamate transport[48]. The maintenance of extracellular potassium ion (K^+) seems to be tightly regulated by astrocytes[49] by specific ion channels, and dysfunction of these ion channels is correlated with Rett's syndrome and Huntington's disease[50,51]. Several ion channels like potassium channels are highly expressed in astrocytes, allowing these cells to control ionic environment.

1.2.1. The concept of the tripartite synapse

As it was mentioned before, astrocytes communicate closely with neurons mainly through release and uptake of substrates. Astrocytes are able to release gliotransmitters upon neuron stimulation, maintain ionic environment, control of the supply of neurotransmitters in the synapses, among others. Such properties make astrocytes not only able to modulate synaptic transmission but essential for it.

It is this astrocytic mediated-modulation of synaptic transmission that leads to the concept of the tripartite synapse. This concept claims that there is a bi-directional neuron-astrocyte communication within the synapses where astrocytes can receive information from presynaptic and postsynaptic terminals (by sensing neurotransmitters and/or transmitters released by neurons through specific transporters and/or receptors expressed in the astrocytic plasma membrane) and respond to this information (by releasing gliotransmitters), therefore regulating synaptic information. It is called tripartite synapse because there a communication between presynaptic terminals, postsynaptic terminals and astrocytes. Even though astrocytes do not have the ability to trigger action potentials, they exhibit a unique form of excitability through variations in intracellular calcium concentration ($[Ca^{2+}]_i$) (see section *Astrocytic Calcium Signalling* for more detail). In the synapse, presynaptic terminals release neurotransmitters that bind to the postsynaptic terminal receptors. In many cases, these neurotransmitters spill-over and also bind to neurotransmitter receptors in astrocytes (Figure 1.3). This binding increases $[Ca^{2+}]_i$ in astrocytes triggering transmitter release, such as ATP, glutamate, and D-serine from astrocytes themselves (denominated gliotransmitters). These then bind to receptors in the presynaptic terminal modulating synaptic transmission. Glutamate released from astrocytes evokes slow inward currents by activating postsynaptic NMDARs[52,53]. Beyond excitatory neurotransmission, astrocytes are also able to release ATP (which then degrades to adenosine), which has an active role in long term depression (LTD)[54]. Gliotransmission is therefore an important mechanism for the control of synaptic transmission.

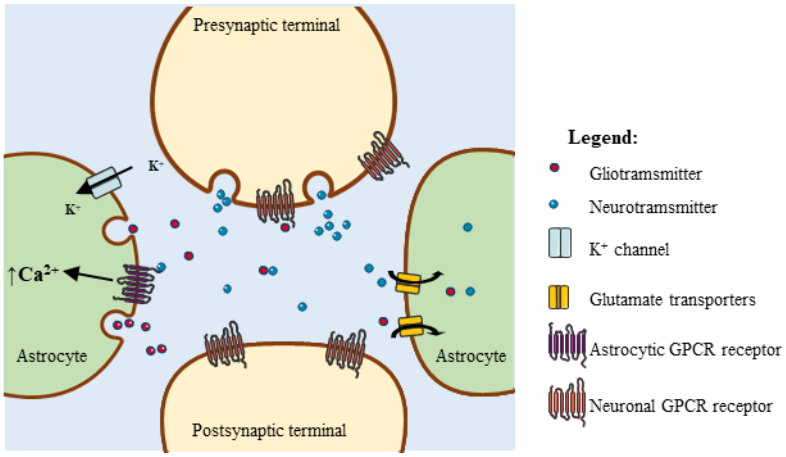

Figure 1.3-Tripartyte Synapse.
Astrocytes are able to receive information from neurons through the activation of astrocytic GPCRs, whose activation triggers $[Ca^{2+}]_i$ elevation. This calcium signalling then causes astrocytes to respond to neuronal stimulation through release of gliotransmitters which then bind to neuronal GPCRs in both pre- and postsynaptic terminals. Beyond that, astrocytes are also able to regulate extracellular levels of potassium through specific ion channels and have a pivotal role in glutamate transport, protecting neurons against excitotoxicity.

1.2.2. Astrocytic Calcium Signalling

Calcium ion (Ca^{2+}) is a ubiquitous second messenger that impacts nearly all aspects of cellular life, being able to bind to thousands of proteins. This ion has a wide-range of physiological functions such as muscle contraction, neurogenesis, and oxidative stress. Beyond that, high concentrations of intracellular calcium can lead to cell apoptosis (for review see [55]). These changes can be caused by Ca^{2+} regulation of enzymatic activity, ion pumps, and other components of the cytoskeleton.

A universal mechanism for Ca^{2+} signalling is the release of this ion from intracellular stores. This mechanism is usually triggered by activation of a G-protein coupled receptor, primarily $G_{q/11}$ subtypes which lead to activation of PLC cleaving phosphatidylinositol 4,5 bisphosphate (PIP_2) into 1,4,5-inosotol triphosphate (IP3) and DAG. IP3 then binds to IP3 receptor (IP3R) in the endoplasmic reticulum

(ER) allowing the calcium to diffuse from the ER to the cytosol. This event increases the $[Ca^{2+}]_i$ from around 100 nM up to 1 µM for a variable amount of time. These types of IP3-dependent transients can have a broad range of periodicities, from 10 to 400 seconds. However, not much is known about the control of these mechanism that might be both cell and/or ligand dependent.

Astrocytes express a variety of ion channels and receptors which can be activated upon neuronal activity eliciting increases in $[Ca^{2+}]_i$, triggering release of gliotransmitters. Activation of different receptors and/ or channels generate intracellular Ca^{2+} release from common intracellular stores and the decline of Ca^{2+} transients upon continuous stimulus is due to receptor desensitization rather than Ca^{2+} depletion from calcium stores, since these can be rapidly refilled[56]. Ca2+ signalling in astrocytes is very broad. Ca2+ signals elicited from GPCR activation in astrocytes can have different ranges from local microdomains responsible, for example, for the regulation of neurotransmitter release, postsynaptic plasticity, to global Ca^{2+} signals that can lead to a propagating Ca^{2+} waves, modulating neuronal activity (for review see [57]). Besides being able to respond to external stimulation with intracellular calcium elevations, astrocytes can also transmit these signals to adjacent/neighbor cells as calcium waves either by direct mobilization of calcium through the ion channels in gap junctions[58] or through "*de novo*" generation of messengers in the extracellular space which then bind to receptors of neighboring cells leading to a calcium increase in these[58]. These mechanisms are not mutually exclusive. Astrocytic calcium waves are able to modulate neuronal activity and neuronal activity has been shown to trigger astrocytic calcium waves[59], indicating how astrocytic calcium signalling is an essential component of the neuron-astrocyte communication at the synapse. In the hippocampus, the astrocytic calcium-mediated glutamate release activates mGluRs in presynaptic terminals leading to an increase in neuronal intracellular calcium, increasing the probability of neurotransmitter release in CA1 neurons[60]. In the cortex, astrocytic CB1R-mediated calcium intracellular increases lead to glutamate release which then activates pre-synaptic NMDA receptors inducing t-LTD[40]. Furthermore, the astrocytic glutamate release is also able to activate directly postsynaptic mGluR[39,61]. Astrocytes are also able to generate intrinsic calcium waves in the absence of external signals/stimulations from neurons[62].

In summary, astrocytic calcium elevations precede the release of gliotransmitters such as glutamate or ATP which may ultimately modulate synaptic transmission.

1.3. Glutamate and the glutamate-glutamine cycle

Classified as a neurotransmitter in the 1950's[63], glutamate is the main excitatory neurotransmitter in the mammalian brain, having a pivotal role in neuronal signalling and being implicated in many other brain functions such as cognition, memory, and learning. Within the CNS, glutamate binds to receptors coupled to ionotropic channels such as NMDAR, AMPA, and kainite and metabotropic receptors such as mGluR1 2, 3, 4, and 5. Beyond that, glutamate also plays a key role in the CNS development like synapse induction and migration[64].

In the CNS, glutamate can be taken up by cells for metabolic purposes or to be used as a neurotransmitter. In the nerve terminals, glutamate is stored in synaptic vesicles through a vesicular glutamate transporter, being released by exocytosis in the synaptic cleft upon a stimulus in a calcium-dependent manner. In the synaptic cleft, glutamate bind to glutamate receptors found in the postsynaptic terminal, whose activation elicit influx of positively charged ions like sodium or potassium resulting in the depolarization of the neuron. The glutamate that does not bind to the glutamate receptors either is diffused in the extracellular space or is taken up by glutamate transporters which are localized mainly in the astrocytes[48]. Once in astrocytes, glutamate is converted into glutamine via glutamine synthetase (GS) in an ATP-dependent manner. Glutamine is then released in the extracellular space to be taken up by nerve terminals by specific transporters, where is reconverted into glutamate to be re-used as a

neurotransmitter. This trafficking of glutamate and glutamine is known as the glutamate-glutamine cycle. However, glutamate is not only used as a neurotransmitter, being used for metabolic purposes such as protein synthesis, energy metabolism, among others. In fact, glutamine obtained by neurons from astrocytic origin is a major carbon source for neurons[65]. It also plays a role in brain development, possibly through Ca^{2+} facilitation[66].

1.3.1. Glutamate uptake

One of the main functions of the astrocytes and neurons is neurotransmitter uptake that is crucial for the normal function of synaptic transmission. During synaptic transmission, neurotransmitters are released in the synaptic cleft by presynaptic terminals, in a calcium dependent way, then binding to receptors present in the postsynaptic terminals, leading to the propagation of the signal. Although neurotransmitters are essential for synaptic transmission, they must be kept at a low concentration to prevent excitotoxicity, and for that they must be cleared from the synaptic cleft once their role has been fulfilled. This can be achieved either by diffusion of the neurotransmitters from the synaptic cleft, their degradation in the synapse cleft, or their uptake from the synapse by surrounding cells. For this purpose, both neurons and astrocytes express specific transporters for these transmitters that allow for the recycling and the regulation of the concentration of neurotransmitter present in the synapse to control how long a signal resulting from the neurotransmitter lasts.

Because glutamate, besides being a neurotransmitter, is also a potent neurotoxin[67], its clearance from the synaptic cleft after it has performed its function is crucial from maintenance of homeostasis of CNS. Glutamate uptake can prevent excitotoxicity, an event where neurons are damaged or even killed by the overactivation of receptors such as AMPA or NMDA. Excitotoxicity is implicated in many neuronal diseases, such as epilepsy, Parkinson's disease[68], and Alzheimer's disease[69,70], which only emphasizes the importance of a tight regulation of glutamate uptake. To prevent this process, most brain cells such as neurons and glial cells have mechanisms to incorporate glutamate, preventing it from over activating receptors expressed in postsynaptic terminals. Glutamate clearance can be performed in two different processes: high-affinity sodium dependent uptake and low-affinity sodium independent uptake, although high-affinity sodium dependent uptake has been shown to have a bigger role in glutamate uptake[71]. High-affinity sodium dependent uptake is achieved by specialized proteins that work as glutamate specific transporters. These proteins can clear glutamate from the extracellular space into the cells where it can be metabolized or recycled according to the cell's needs. So far, five high-affinity glutamate transporters have been identified in the cells of the nervous system: GLAST, GLT-1, EAAC1, EAAT4 and EAAT5. Sodium-dependent transport is driven by the electrochemical gradients across the cell membrane and both sodium and hydrogen ions are required for the uptake. Beyond that, it is an electrogenic process (positive charge moving in). While neurons perform glutamate uptake, astrocytes are essential to the normal function of this task[72]. Modulation of glutamate transport can be associated with a variety of factors such as DNA transcription, mRNA splicing and degradation, protein synthesis and targeting, amino acid transport activity, and associated ion channel activities. For example, GLT-1 has been shown to have a very dynamic membrane diffusion which modulates synaptic transmission[73].

1.3.2. Astrocytic GLAST and GLT-1 glutamate uptake

The glutamate transporters Glutamate Aspartate transporter (GLAST) or excitatory amino acid transporter 1 (EAAT1) and glutamate transporter (GLT-1) or Excitatory amino acid transporter 2 (EAAT2) are the main responsible for glutamate transport and, even though they differ in the acronym, these proteins do not have functional differences between them. Both proteins perform sodium and hydrogen coupled transport of L-glutamate as well as L- and D-aspartate (Figure 1.4). Originally cloned

from rat[74,75], human homologues have also been cloned[76,77] and present about 65% similarity in an amino acid level. There has been some debate as to which transporter has more impact in glutamate uptake. It is important to mention that, even though it is an agreement that both transporters are important, their expression through the CNS is not uniform. GLT-1 has a more prominent expression in the hippocampus[78], while GLAST expression is more pronounced in the cerebellum. Within each area, there is debate especially for GLT-1 where is localized cellularly. While in the hippocampus, inhibition of astrocytic GLT-1 leads to cease of glutamate transport[79] defending astrocytic GLT-1 is the main responsible for glutamate uptake, in synaptossomes, the kinetic constant maximum velocity of transport (V_{max}), was significantly decreased when neuronal GLT-1 was knocked out[80], suggesting that pre-synaptic GLT-1 has a major role in glutamate uptake.

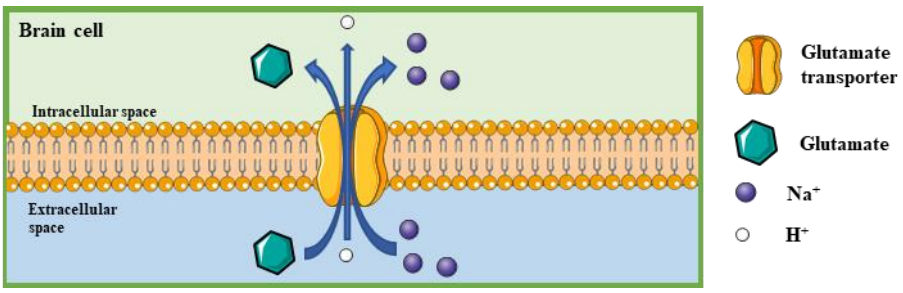

Figure 1.4- Stoichiometry of glutamate transporters.
Per each molecule of glutamate, glutamate transporters also carry about 3 ions of sodium and one ion of hydrogen.

1.4. Adenosine Receptors in Astrocytes

Similar to CB1R, adenosine receptors comprise a group of GPCR proteins that are activated upon binding of adenosine. To this date, four subtypes of adenosine receptors have been discovered and cloned: A_1, A_{2A}, A_{2B} and A_3, all seven transmembrane domain G protein coupled receptors[81]. The ligand for these receptors, adenosine, is an ubiquitous purine nucleoside metabolized from ATP in the organism that exerts many type of brain functions such as sleep regulation[82]. The expression of these receptors occurs throughout the organism but is not uniform. Adenosine A_1 and A_{2A} receptors expression is predominant in the brain, while adenosine A_{2B} and A_3 receptors are more expressed in peripheral tissue such as aortic vascular smooth muscle, large intestine, urinary bladder, lungs, heart, among many others[81]. Adenosine A_1 and A_3 receptors are often inhibitory being coupled to $G_{i/o}$ protein, inhibiting cAMP accumulation and PKA activity, while A_{2A} and A_{2B} are usually coupled to G_s protein and when activated enhance cAMP accumulation and PKA activity.

In the CNS, adenosine is considered a neuromodulator and exerts its function by binding to A_1R and A_{2A}R. Activation of this receptors has been associated with critical brain functions such as neurotransmitter release[83], neurotransmitter uptake[84–86], and synaptic plasticity[87]. More specifically, astrocytes release ATP which then degrades into adenosine. Extracellular adenosine then binds to the adenosine receptors. Activation of astrocytic A_{2A}R decreases aspartate uptake[86] and regulates memory[88], while astrocytic A_1R are correlated with PLC activation[89], sleep[90], and growth factors[91].

1.4.1. Cross-talk between CB1 receptors and adenosine receptors in the brain

Both endocannabinoid and adenosergic systems are important modulators in brain function, whose receptors are present in both neurons and astrocytes. Many studies report that these systems do not work independently having an interaction and/or heterodimerization between CB1Rs and adenosine A_{2A}R, mainly in neurons. Presynaptic adenosine A_{2A}R diminish CB1R effect in glutamatergic transmission[92] and the majority of glutamatergic terminals that express adenosine A_{2A}R also co-express CB1R,

implying that in most cases their activity depends on adenosine $A_{2A}R$. As for adenosine A_1R, these receptors have shown to attenuate CB1R-mediated inhibition of glutamate and GABA release in the hippocampus leading to an enhancement of the disruptive effect of THC on short-term spatial memory task[93,94]. There are no reports of interaction between adenosine receptors and CB1R in astrocytes.

2. Aims

Astrocytes play a key role in synaptic transmission, by interacting directly and indirectly with pre- and postsynaptic terminals and surrounding astrocytes. Neurotransmitter transporters are essential for the normal function of the brain and are present in neural and glial cells. These proteins need to continuously go through a fine control since abnormal activity of these proteins has been correlated with several neurodegenerative diseases. GLAST and GLT-1 are the main glutamate transporters in astrocytes and are modulated by various cellular components like calcium signalling, a main way of communication in these types of cells.

On the other hand, the endocannabinoid system is a modulatory system whose activity is able to modulate activity of many components involving the brain, such as synaptic transmission. In astrocytes, it has been stated that CB1R activation elicits calcium elevations. Beyond that, interaction between adenosine A_{2A}Rs and CB1Rs has been extensively studied at neuronal level but never in astrocytes.

Therefore, the general aim of this work is to study the cannabinoid calcium-mediated signalling in astrocytes and to observe its relevance upon glutamate transporters activity. To do so, calcium imaging and glutamate uptake assays will be performed in primary culture of astrocytes, and the following specific aims will be pursued:

1. To evaluate CB1R, GLAST, and GLT-1 expression in primary culture of astrocytes;
2. To study CB1R activation upon calcium signalling and glutamate transport in primary culture of astrocytes; to establishe a possible correlation between calcium signalling, mediated by CB1R activation, and glutamate transporters;
3. To study the crosstalk between adenosine A_{2A}Rs and CB1Rs in astrocytes at calcium signalling level.

3. Methods

3.1. Drugs and Antibodies

The following antibodies were used: goat anti-guinea pig or rabbit IgG conjugated to Alexa Fluor 488 (Santa Cruz Biotechnology), polyclonal antibody anti-CB1, rabbit polyclonal anti-glial fibrillary acidic protein (anti-GFAP); guinea pig polyclonal anti-GLT-1(Japan) and rabbit anti-GLAST (Abcam, Cambridge UK)

The following drugs were used: L-[3,4-^{3}H]-Glutamic Acid([^{3}H] Glutamate, specific activity 40–80 Ci/mmol) from Amersham (Buckinghamshire, UK) ; L-Glutamic acid monosodium salt hydrate (Glutamate) and ethyleneglycol bis(β-aminoethylester)-N,N,N',N'-tetraacetate (EGTA) were obtained from Sigma (St. Louis, USA); acetoxymethyl bis(O-aminophenoxy)ethane-N,N,N',N'-tetraacetate(BAPTA-AM) was obtained from Molecular Probes (Eugene, OR, USA); Arachidonyl-2'-chloroethylamide (ACEA), α-Cyclopiazonic Acid (CPA), 2-[1-(3-dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl) maleimide (GF109203X), 2-Amino-5,6,7,8-tetrahydro-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-4H-chromene-3-carbonitrile (UCPH-101), 2-Amino-5,6,7,8-tetrahydro-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-4H-chromene-3-carbonitrile (WAY-213613), 2-(2-

FURAnyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 58261), 4-[2-[[6-Amino-9-(N-ethyl-β-D-riboFURAnuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS 21680), (R)-Adenosine, cyclic 3',5'-(hydrogenphosphorothioate) triethylammonium (cAMP-Rp), Pertussis toxin (PTx) and 1-6-[[(17$_\beta$)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) were purchased from Tocris (Avonmouth, UK); 5-Oxazolecarboxylic acid, 2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-(2-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzoFURAnyl)-, (acetyloxy)methyl ester 108964-32-5 (FURA-2AM) was obtained from Thermo Fisher (Massachusetts) . Stock solutions of drugs were prepared with dimethylsulphoxide or distilled water or ethanol and kept at -20^{o}C until used. Drugs' solutions were prepared from stock solutions diluted in either culture medium and/or incubation buffer immediately before use.

3.2. Primary astrocytic cultures

Animal handling and experiments were conducted according to the guidelines set in Directive 2010/63/EU of the European Parliament ant the council of the European Union. Primary cortical astrocytes were obtained from 0-2 days old Sprague Dawley (Charles River, Barcelona, Spain) pups of either sex as described by Sandra H. Vaz *et al*. (2011) [95]. Pups were sacrificed by decapitation and the heads were sterilized by submersion in alcohol three times. The brains of the pups were dissected in phosphate-buffered saline (PBS, in mM: 137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.2H_2O$ and 1.5 mM KH_2PO_4, pH 7.4) out by removing the scalp covering the top part of the skull. A mid line incision was made in the skull exposing the brain which was then dissected out by introducing a spatula between the cortex and olfactory bulbs separating them and sliding the spatula under the brain cutting the optical nerves and removing the brain from the skull. The cerebellum was removed, and the hemispheres were separated. The basal ganglia, meninges, and hippocampal formations were removed, leaving only the cortex area. The cortexes were then dissociated mechanically with a 10 mL pipette in 4.5 g/L glucose Dulbecco's Modified Eagles Medium (DMEM, Gibco, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Paisley, UK), 0.01% antibiotic/antimycotic (100 U/mL penicillin, 0.25 µg/mL anphotericin B and 100 µg/mL streptomycin, Sigma, Steinheim, Germany) solution and 0.01% glutamine (Gibco, Paisley, UK) at 37 ^{O}C.

The dissociated cells were filtered through a 230 µm pore mesh and a 70 µm mesh (BD Falcon, Erembodegem, Belgium). After each filtration, the cells underwent through two centrifugations at 200 g × 10 minutes at room temperature (RT). After each centrifugation, the supernatant was discarded, and the pellet was resuspended in 10 mL of fresh DMEM supplemented medium. In the last centrifugation, the pellet was resuspended for the final volume of 10 mL supplemented medium for each cortex. Cells were plated in 24-well culture plates for the uptake experiments and plated in a T-75 flask for calcium experiments and immunocytochemistry.

To reduce the microglia contamination, cultures underwent horizontal agitation[96]. After 6 days in culture (DIC), the T-75 flasks were agitated horizontally at 300 rpm for 4-5 hours and on DIC7 both 24-well plates and T-75 flasks were agitated at a 300 rpm for 4-5 hours. T-75 flasks were then replated in an 8-well plate for the calcium imaging experiments in a dilution of 1 mL of cells plus 7 mL of modified DMEM. For the immunocytochemistry assays, the T-75 flasks were replated in 24-well plates covered with a glass coverslip in 1/11 mL dilution.

During incubation, cells were kept in a humified atmosphere (5% CO_2) at 37 ^{O}C. Culture medium was changed on DIC 3 and every 7 days after culture.

3.3. Glutamate uptake assay in primary astrocytic cultures

Determination of glutamate uptake was obtained as described by Leonova *et al*. (2001)[97] with slight modifications. As it was mentioned before, glutamate uptake was performed in 3-week astrocytes cultures seeded in 24-well plates. Before the transport assay, astrocytes were pre-incubated for 3-5 hours at 37 ^{O}C (5% CO_2) in serum-free 1g L $^{-1}$ glucose DMEM. Following pre-incubation, cells were rinsed once in serum-free DMEM and allowed to equilibrate for 5 minutes before drug incubation. The drugs were then administrated at showed time.

For the concentration curves, transport was initiated by addition of 10, 30, 60, 90, 120, 200, 400, and 1000 µM of glutamate for 4 minutes. Once the value of the kinetic constants affinity (K_m) and V_{max} were established, transport was initiated by addition of 200 µM of L-[2.3-^{3}H]-glutamate(Perkin Elmer, UK) for 4 minutes at 37 ^{O}C in a transport buffer (in mM: 137 NaCl, 5.4 KCl, 0.4 $MgSO_4$, 0.4 $MgCl_2$, 1.26 $CaCl_2$, 0.64 KH_2PO_4, 3 $NaHCO_3$, 5.5 glucose and 20 HEPES, pH 7.4). Transport was terminated by rapid removal of radioactive medium and two washes with ice cold transport buffer. The cells were then harvested in 200 µL lyses buffer (0.1% Triton X-100 + 100 mM NaOH) for 1 hour. The amount of L-[2.3-^{3}H] glutamate taken up by astrocytes was quantified by liquid scintillation counting. GLAST mediated glutamate uptake was taken as the difference between the L-[2.3-^{3}H] glutamate in the absence and in the presence of GLAST blocker UCPH-101 (10 µM). GLAST blocker was added five minutes prior to the other drugs (CPA, U73122, GF109203X, AM251, CGS 21680, SCH, PTx, and BAPTA-AM). ACEA was added at minute 20 of incubation, where its effect was observed for 20 minutes.

For time course assays, 100 µM of L-[2.3-^{3}H]-glutamate were added for 1, 2, 3, 4, 6, 8, 12, 14, and 16 minutes to observe for how long the transport remained linear.

Scintillation counts per minute were normalized by total amount of protein in each replicate. Total protein was quantified with Bio-Rad DC reagent (Hercules, CA, USA), using BSA (Bovine Serum Albumin) as the standard protein to establish the calibration curves.

3.4. Calcium Imaging

For the calcium imaging experiments, 7 DIC astrocytes kept in T-75 flasks were plated in 8 well Ibidi plates with a glass bottom (ibidi GmbH, Martinsried, Germany) with 8 wells of 9.4 x 10.7 x 6.8 (mm). These wells were previously coated with Poly-d-lysine (PDL) for 1 hour and washed 3 times with sterile water. For Calcium Imaging Experiments, astrocytes had to be seeded in a plate with a glass bottom and due to their poor adherence to glass surfaces a coating procedure had to be performed. This procedure was already optimized in for astrocytic cultures in our lab. The plating of 18-23 DIC astrocytes in 8 well Ibidi plates was made by trypsinization (1% trypsin-EDTA) for 1-2 minutes under agitation and this reaction was stopped by the addition of 4.5 g/L glucose DMEM containing 10% fetal bovine serum with 0.01% antibiotic/antimycotic and glutamine. Until the day of the experiments, the plated cells were maintained in a suitable growing medium in a humidified atmosphere (5% CO_2) at 37 ^{O}C. At the day of the experiment, the cell medium was removed, and the cells were washed three times with external physiological solution Ca^{2+}-HEPES buffer (composition in mM: NaCl 125, NaH_2PO_4 1.25; KCl 3; D(+)-glucose 10 $CaCl_2$; $MgCl_2$ and HEPES 10; pH 7.4 adjusted with NaOH). Prior to the experiment, cells were loaded with sensitive calcium fluorescent dye FURA 2-acetoxymethyl, FURA-2AM (5µM), for 45 minutes. Using a ratiometric dye has some advantages that other assays like single wavelength probes cannot offer like ratio signal is independent of the dye concentration, light intensity. These characteristics allow the evolution of intracellular calcium to be determined independently of these artefacts. After FURA-2AM incubation, the cells were washed once again with Ca^{2+}-HEPES buffer, 3 times. The ibidi plates were mounted on an inverted microscope (Axiovert 135TV, Zeiss) with a xenon

lamp and band-pass filters of 340 and 380 nm wavelengths. Throughout the experiment, cells were kept at 37 ^{O}C in a humidified atmosphere. For the first 5 minutes of the assay, a calcium baseline was established, and the tested drugs were applied directly in the medium. The CB1R agonist was applied 15 minutes after the antagonist in the same conditions. In all experiments, at 40 minutes, ionomycin (2 µM) was added. This calcium ionophore in high concentrations can induce necrotic cell death[98] by a substantial increase of intracellular calcium. This step allowed to recognize which cells were viable during the assay. Only cells that responded to ionomycin through a substantial increase of ratio 340/380 nm were considered for statistical analysis. Image pairs obtained every 5-10 seconds by exciting the preparations at 340 and 380 nm which were then used to obtain ratio images. Excitation wavelengths were changed using a high-speed wavelength switcher, Lambda DG-4 (Sutter Instrument, Novato, CA, USA), and the emission wavelength was set to 510 nm. Image data were recorded by a CCD camera (Photometrics CoolSNAP fx) and processed and analysed by software MetaFluor (Universal Imaging, West Chester, PA, USA). Regions of interest were obtained by defining the profile of the cells and averaging the fluorescence intensity within the delimited area. Intensity values were converted to ratio 340/380 nm and the all the values were normalized by the first ratio of each cell.

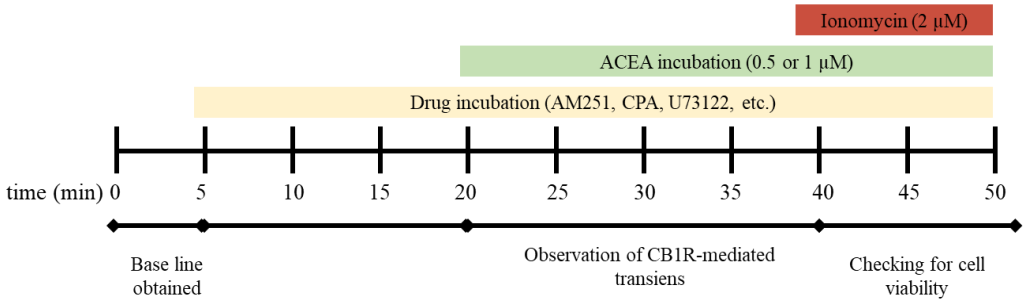

Figure 3.1 – Scheme of the calcium imaging protocol.
Representative time course of calcium experiment. In the first 5 minutes there is no exogenous application to obtain a basal line of physiological conditions. At the fifth minute, a specified drug was applied exogenously to activate or inhibit a certain candidate implied in the CB1R calcium signalling. After 20 minutes of incubation, cells were incubated with ACEA at a specific concentration. At minute 40, ionomycin was exogenous applied to check for cell viability. Only cells that had a substantial increase of ratio 340/380 nm were select for posterior analyses.

3.4.1. Calcium Imaging Analysis

The frequency and amplitude of calcium transients were determined with MATLAB and Statistics Toolbox Release 2017b, The MathWorks, Inc (Natick, Massachusetts, United States.) with a specific script. For the first five minutes of the experiment a basal line was calculated. A mean and S.E.M. of all recorded ratios per each cell was obtained. Transient validation was performed as described by Horvat *et al.* (2016)[99] with some modifications. Cells that had ratio transients during the first 5 minutes of the experiment were discarded from statistical analysis. For a transient to be considered valid, some criteria had to be fulfilled: only if the ratio was higher than mean plus 15 times the S.E.M. it would be considered for a transient. Beyond that, the normalized ratio should be above this margin longer than 10 seconds and less than 700 seconds. If the final transient is still occurring at minute 40, a special consideration was acquired for transient validation. Only transients that were in the descending phase at this time were considered. For each region of interest, the peak of each transient as well as the occurrence of transients were recorded.

3.4.2. Statistical Analysis

Statistical analyses of Ca^{2+} imaging data were performed using GraphPad Prism 5 (San Diego, CA, USA) software. Data are expressed as the Mean ± SEM., and two-sample comparisons were made using

Student's *t*-tests and multiple comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni post-test.

3.5. Immunocytochemistry assays

At DIC 7, the cells from the T-75 flasks were plated into 24-well plates in a 1/11 mL dilution, with each well containing a glass coverslip coated with Poly-d-lysine (10 µg/mL) as described in *Astrocytic cortical culture section.*

For cell fixation, cells at DIC 21-23 were washed twice for 5 minutes in PBS pH 7.40 and fixed with 4% paraformaldehyde (PFA) for 15 minutes at RT. To prepare for the immune assays, cells were washed twice with PBS. To clear up PFA residues, cells were rinsed for 10 minutes at RT with freshly prepared 0.1 M glycine in PBS. The cells were then permeabilized with freshly prepared 0.5% Triton X-100 in PBS for 10 min at RT and the washed twice with PBS. To decrease unspecific interactions, cells were incubated in a blocking solution (PBS containing 10% FBS for 1 hour at RT). The glass coverslips containing the cells were then removed from the 24-well plate and placed (cells side down) in a 20 µL drop containing the primary antibodies diluted to the optimized concentration in blocking solution overnight at 4 ^{O}C. The glass coverslips were then transferred back to the 24-well plate (cells side up) and washed three times for five minutes with PBS containing Tween 80 (20%, PBS-Tw). The glass coverslips were incubated for one hour at RT in a 20 µL drop containing the secondary antibodies diluted to the optimized concentration in blocking solution and washed three times with PBS-Tw for 5 minutes. A 20µl drop of Hoechst 33342 solution (1:100 dilution from 2 mM stock) on top of each coverslip for five minutes at RT. The Hoechst 33342 was aspirated, and the glass coverslips were washed three times with PBS-Tw for ten minutes. Once dried, the glass coverslips were carefully mounted (cells side down) in a small drop (4 µL) of Mowiol (Sigma, Steinheim, Germany) on a slide and dried for 24 hours at RT. Then the slides were kept at 4 ^{O}C.

Images were acquired using a Zeiss (Thornwood, NY, USA) LSM 710 microscope.

For CB1R and GLT-1 localization in astrocytes, cultures were incubated for 90 minutes with the primary antibodies guinea pig anti-CB1R (1:200) and mouse anti-GFAP (1:400) or mouse anti-GLT-1 (1:200) and mouse anti-GFAP (1:400). For GLAST localization, primary antibodies glutamate transporters localization, rabbit anti-GLAST (1:200) and mouse anti-GFAP (1:400). Visualization of GFAP, CB1R, GLT-1 and GLAST positive-cells was accomplished upon 1 hour incubation with anti-guinea pig IgG conjugated to Alexa Fluor 488 (1:500) for CB1R; goat anti-mouse or anti-rabbit IgG conjugated to 568 (1:1000) for GFAP; goat anti-mouse IgG conjugated to Alexa Fluor 488 (1:500) for GLT-1, and goat anti-rabbit conjugated to Alexa Fluor 488 (1:500) for GLAST.

In negative controls, cells were incubated with a primary antibody of one component and with the secondary antibody of a different component. This allows to observe not only if there is unspecific bonding between antibodies that are not supposed to bind but also to verify if immunoassays had a significant amount of autofluorescence.

4. Results

4.1. Glutamate transporters and CB1R expression in cortical culture of astrocytes

In order to evaluate CB1R and glutamate transporters, GLAST and GLT-1, expression on 21-23 DIC enriched astrocytic cultures, an immunocytochemistry assay was performed. GLAST and GLT-1

expression in astrocytes had already been confirmed, although this expression varies in different stages[97,100]. CB1R expression has been proven by immunocytochemistry in the astrocytes from hippocampus[39] but not in astrocytes from the cortex. For that reason, the CB1R expression in cultured cortical astrocytes was accessed. These type of cultures are highly enriched in astrocytes (being around 95% GFAP-positive) making them suitable for the study of astrocytes[101]. Astrocytes exhibited immunoreactivity for CB1R confirming its presence in primary cortical astrocytes (Figure 4.1). As for glutamate transporters, astrocytes also showed immunoreactivity towards these two proteins (Figure 4.2). Both GLAST and GLT-1 were expressed in astrocytes. GLAST appeared to have a higher expression in intracellular and membrane. On the other hand, GLT-1 seemed to have a higher expression in the area surrounding the nucleus.

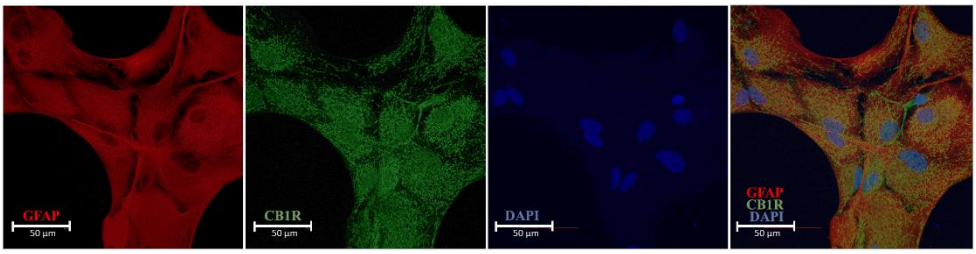

Figure 4.1- CB1R expression in astrocytic cultures.
Presence of CB1R on cultured astrocytes (21-25DIC). Nuclei were stained with Hoechst, GFAP stained astrocytes are red and CB1R immunoreactivity is stained green. Immfluorescence images were acquired with a 40x objective in a Zeiss (Thornwood, NY, USA) LSM 710 microscope. Scale bars, 50µm.

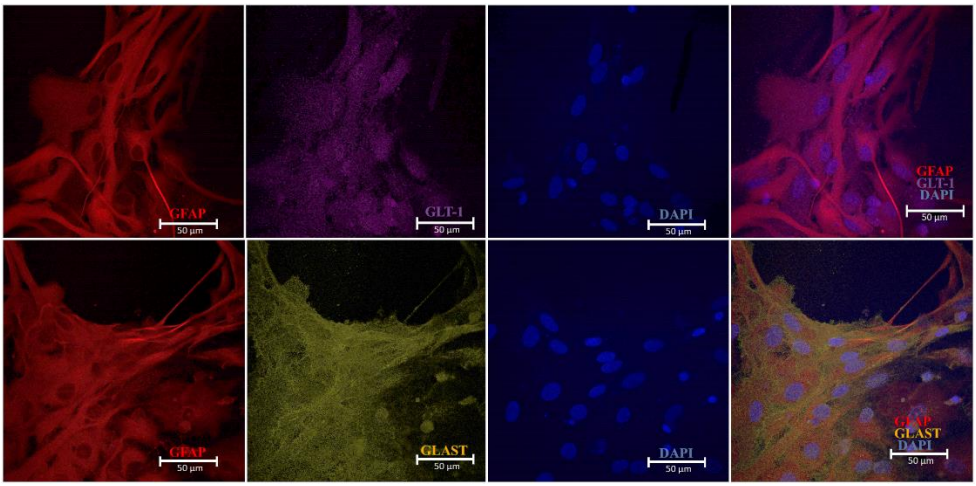

Figure 4.2- GLT-1 and GLAST expression in astrocytic cultures.
Presence of glutamate transporters, GLT-1 and GLAST on cultured astrocytes (21-25DIC). Nuclei were stained with Hoechst, GFAP stained astrocytes are red and GLT-1 and GLAST immunoreactivity is stained purple and yellow respectively. Immfluorescence images were acquired with a 40x objective in a Zeiss (Thornwood, NY, USA) LSM 710 microscope. Scale bars, 50µm.

4.2. CB1R-mediated calcium signalling in astrocytes

4.2.1. CB1R activation in astrocytes leads to Ca^{2+} transients whose amplitude is concentration dependent

It has been previously reported that activation of CB1R triggers elevations in $[Ca^{2+}]_i$[39]. To better understand this mechanism, the CB1R effect on calcium signalling was evaluated by using the calcium imaging technique as it was described in section *Calcium Imaging*. 18-23 DIC astrocytes were incubated with FURA-2AM, a membrane cell dye that binds to calcium allowing to observe intracellular calcium

evolution through time. In the first five minutes of the experiment, a basal line of calcium ratio was obtained. To activate CB1R, 20 minutes after the beginning of the experience, cells were incubated with ACEA (1 µM), a synthetic cannabinoid highly selective to CB1R. To check cell viability, at minute 40 of the experiment, astrocytes were incubated with ionomycin (2 µM), a calcium ionophore. If the cells were viable, then ionomycin incubation would lead to an intracellular calcium increase which, in this type of experiment, would be revealed as an increase of the ratio 340/380 nm. Every cell that did not have a substantial increase of the ratio upon ionomycin incubation was discarded from the experiment and posterior statistical analysis. To make sure the Ca^{2+} transients weren't elicited by changes in superficial tension, sham controls were performed. In these condition, at 5 and 20 minutes of experiment, cells were incubated the medium without any drugs. In the sham control, no transients were detected during the all experiment (Appendix 3 - Figure 7.2.A).

ACEA (1 µM) incubation in the cells induced the formation of calcium transients (Figure 4.3 A). In order to quantify the occurrence of these transients, a MATLAB script was made as described in section *Calcium Imaging Statistical Analysis*. In this script, for every region of interest (ROI) it was counted the number of ratio transients per cells and the amplitude of each transient. From the number of transients events, the frequency was obtained has the number of transients per five minutes. When ACEA incubation failed to trigger a transient in a select ROI, the frequency was considered 0 and the amplitude was 1 in order to normalize these values. An average of 0.964 ± 0.109 events per 5 minutes (49 responsive cells from 7 independent cultures) was observed during ACEA (1 µM) incubation and the amplitude of these transients was 1.59 ± 0.03 fold when compared to the basal calcium level.

To better understand the CB1R-calcium mediated effect in astrocytes, a lower concentration of ACEA was tested and both frequency and amplitude were determined in these conditions. Incubation with 0.5 µM of ACEA (24 responsive cells from 3 independent cultures, $P < 0.05$) led to a significative decrease of frequency (ACEA 1 µM: 0.964 ± 0.109 transients per five minutes and ACEA 0.5 µM: 0.554 ± 0.103 transients per five minutes $P< 0.05$) and amplitude (ACEA 1 µM:1.59 ± 0.03 and ACEA 0.5 µM:1.46 ± 0.07) (Figure 4.3.D and E), when comparing both ACEA tested concentrations.

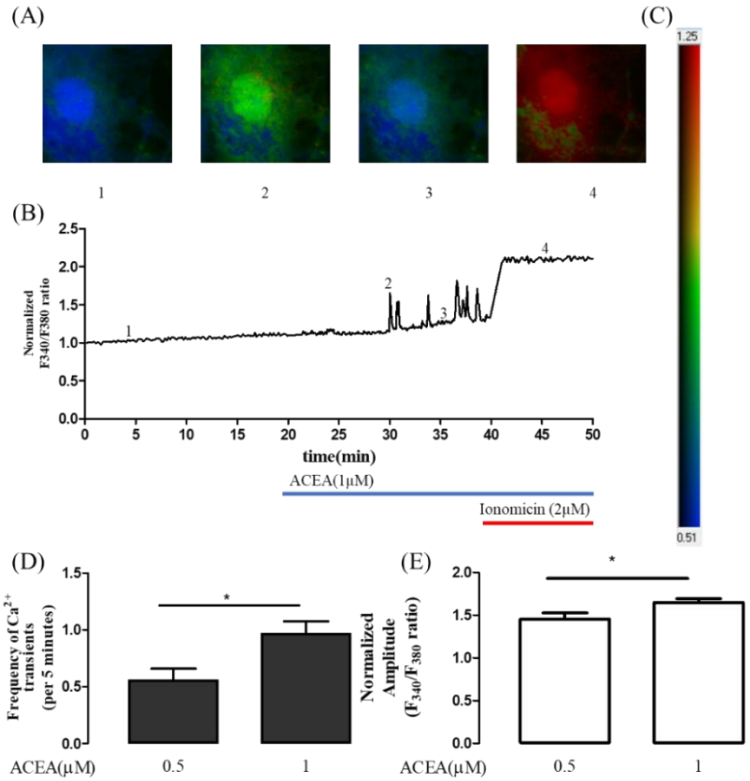

Figure 4.3- **CB1R-mediated calcium signalling in cultured astrocytes.**
(A) Representative fluorescence images of single astrocyte loaded with FURA-2AM and stimulated with ACEA (CB1R agonist) at 20 minutes of experiment. Number in the images correspond with the number in graph (B) indicating the time at which the images were recorded. Application for 20 minutes of ACEA triggered calcium transients. After 40 minutes of experiment, an exogenous application of ionomycin was performed. If astrocytes are viable, incubation of this molecule increases substantially intracellular calcium. Dose-dependent changes in intracellular [Ca^{2+}] transients frequency (D) and amplitude (E) evoked by different ACEA concentrations (0.5 and 1 µM). In both concentrations, ACEA was incubated at 20 minutes of experiment and changes in intracellular calcium was recorded for 20 minutes. Data are expressed as Mean ± S.E.M. of 73 responsive cells from 3-7 independent cultures.; **P* < 0.05 accessed by Student's *t*-test.

The occurrence of these transients was lost (23 responsive cells from 3 independent cultures, $P < 0.001$), when CB1R antagonist AM251 (1 µM) was previously incubated at 5 minutes of the experiment. In these conditions, only one cell with two transients of a total of 23 cells was recorded (Appendix 3-Figure 7.1 B). Therefore, when incubating the cells with AM251 (1 µM) before ACEA (1 µM), it was observed a significant decrease of both frequency (ACEA: 0.964 ± 0.109 transients per 5 minutes and ACEA+AM251: 0.02 ± 0.02 transients per 5 minutes, Figure 4.4. A, $P < 0.001$) and amplitude (ACEA: 1.59 ± 0.03 and ACEA+AM251: 1.09 ± 0.07, Figure 4.4. B, $P < 0.001$) when compared to ACEA (1 µM) alone.

Taking all together, these results suggest that ACEA incubation in cultured cortical astrocytes triggers calcium transients through activation of CB1R expressed in these cells.

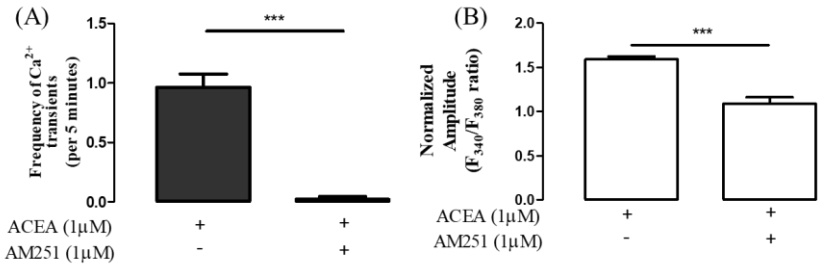

Figure 4.4 **CB1R-mediated calcium signalling in cultured astrocytes.**
(A,B) Incubation of ACEA 1µM lead to a frequency of 0.964 ± 0.109 transients per 5 minutes and an amplitude of 1.593 ± 0.03190 n=7 independent cultures, 49 responsive cells. Prior Exogenous application of CB1R antagonist (AM251, 1µM) 15 minutes prior to ACEA incubation ceases ACEA-meditated calcium transients. Data are expressed as Mean ±S.E.M from a total of 23-49 responsive cells from 3-7 independent cultures assessed by Student's *t*-test.;*** $P < 0.001$.

4.2.2. CB1R-mediated Ca^{2+} transients have intracellular and extracellular origin

The next aim of this work was to evaluate the origin of calcium that led to ACEA-mediated Ca^{2+} transients. Many reports mention that most of astrocytic calcium signalling is due to flux of calcium from intracellular stores within the ER to the cytosol, therefore having an intracellular origin. Because there are also reports of extracellular calcium involved in calcium signalling[101], both propositions were tested. To verify if the calcium flux was originated from intracellular stores, astrocytes were treated with cyclopiazonic acid (CPA, 10 µM) a depletor of intracellular calcium stores prior to the ACEA incubation as mentioned in section *Calcium Imaging*. As it was expected, an increase of calcium basal level was observed after CPA incubation (Appendix 3 - Figure 7.2 D), since this drug elicits an efflux of calcium from intracellular stores to the cytosol. In the presence of CPA (10 µM), ACEA (1 µM) failed to trigger calcium transients, having only just one astrocyte shown 2 calcium transients in a total of 22 cells from 3 independent cultures ($P < 0.001$) leading to a significant decrease of both frequency (ACEA: 0.964 ± 0.109 transients per 5 minutes and ACEA + CPA: 0.02 ± 0.02 transients per five minutes, Figure 4.5. A) and amplitude (ACEA: 1.59 ± 0.03 and ACEA + CPA: 1.01 ± 0.01, Figure 4.5 B). To study the effect of extracellular Ca^{2+} in the occurrence of Ca^{2+} transients, cells were incubated in a low calcium medium (0.5 mM $CaCl_2$ + 1 mM EGTA) throughout the whole time of the experiment. In these conditions, although CB1Rs-mediated calcium transients still occurred (Appendix 3- Figure 7 E), their frequency (ACEA: 0.964 ± 0.109 transients per 5 minutes and ACEA [low calcium]: 0.319 ± 0.109 transients per 5 minutes, Figure 4.5 C, $P < 0.01$) and amplitude (ACEA: 1.59 ± 0.03 and ACEA [low calcium]: 1.16 ± 0.03, Figure 4.5. D, $P < 0.001$) were significantly attenuated. Taken together, these results suggest that CB1R transients triggered by CB1R activation have both intracellular and extracellular calcium components though intracellular calcium stores are essential for the occurrence of these events while CB1R-mediated calcium transients and that extracellular calcium has a significant component in this effect.

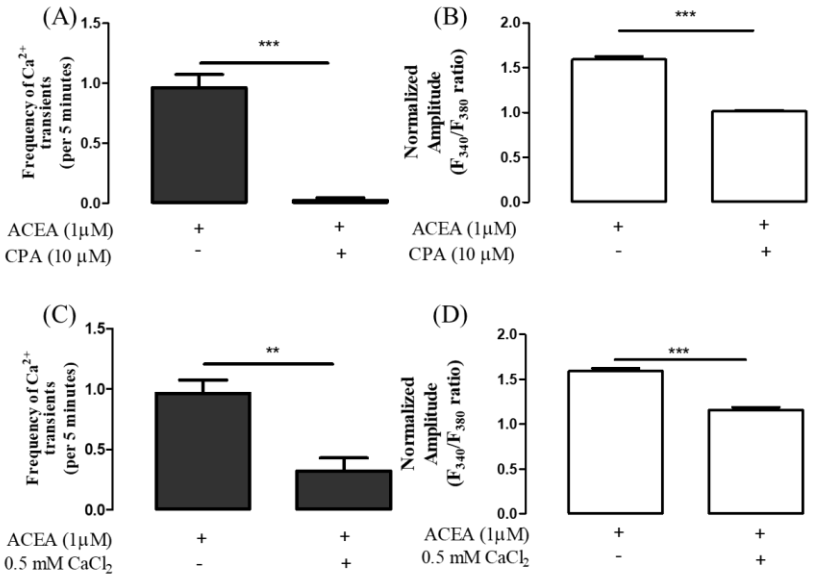

Figure 4.5-**Influence of depletion of intracellular Ca^{2+} stores and low Ca^{2+} medium on the intracellular Ca^{2+} transients mediated by CB1R activation.**
(A,B) Application of CPA (10 µM) 15 minutes prior to ACEA (1 µM) incubation. (C,D) Astrocytes were incubated in low Ca^{2+} medium(0.5mM $CaCl_2$ + 1mM EGTA) for the entire experiment incubating ACEA at 20 minutes of experiment. Data are expressed as Mean ±S.E.M from 18-49 responsive cells from 2-7 independent cultures.; ***P <0.001, **P < 0.01; accessed by Student's *t*-test.

4.2.3. CB1R mediated-calcium transients are Gq/11-PLC-InsP3 pathway-dependent

Usually, CB1R is coupled with $G_{i/o}$ or G_s protein[102]. However, recent studies report that in astrocytes, CB1Rs activation triggers calcium elevations that are PLC dependent, highly suggesting a $G_{q/11}$ coupling to astrocytic CB1Rs[39]. To further inspect the role of PLC in CB1R calcium signalling, a PLC antagonist, U73122 (3 µM), was added prior to ACEA treatment (n=3 independent cultures, 27 responsive cells, P<0.001). When PLC was blocked, ACEA transients were absent in 23 cells out of 27 cells. A significant decrease of frequency (ACEA: 0.964 ± 0.109 transients per 5 minutes and ACEA + U73122: 0.10 ± 0.04 transients per five minutes, Figure 4.6. A, P < 0.001) and amplitude (ACEA: 1.59 ± 0.03 and ACEA+U73122: 1.19 ± 0.05, Figure 4.6. B, P < 0.001) was observed. PLC appears to be essential for the CB1R-mediated calcium signalling, implying that astrocytic CB1Rs are coupled to $G_{q/11}$. Due to previous reports of CB1R coupling to $G_{i/o}$[2] and the fact that some $G_{i/o}$-coupled receptors such as $GABA_B$ receptors are also able to trigger calcium signalling[103], the role of a possible $G_{i/o}$ protein coupled to the astrocytic CB1R had to be considered. With that in mind, cells were incubated with Pertussis toxin (PTx, 5 µg/mL), a $G_{i/o}$ protein antagonist and consequently incubated with ACEA at 20 minutes of experience (n = 3 independent cultures, 21 responsive cells). In the presence of PTx, frequency (ACEA: 0.964 ± 0.109 transients per 5 minutes and ACEA+ PTx: 0.20 ± 0.04 transients per 5 minutes, Figure 4.6 C, P < 0.001) was significantly decreased but there were no significant differences in amplitude (ACEA: 1.59 ± 0.03 and ACEA + PTx: 1.47 ± 0.09, Figure 4.6 D). Although PTx incubation led to an apparent frequency decrease, this could be due to, when incubated with PTx, the presence of ACEA leading to an increase of the basal level together with the occurrence of calcium transients (Appendix 7.2. G). This would lead to the skipping of some Ca^{2+} transients since the MATLAB script uses the basal Ca^{2+} level as a reference to detect Ca^{2+} transients. This has been reported before in HEK cells, where pre-treatment with PTx led to an increase of amplitude of CB1R mediated calcium transients[29]. However, this does

not necessarily mean that it is a $G_{i/o}$ directly interfering with CB1R but some of the $G_{i/o}$ downstream proteins inhibition such as PKA could influence calcium signalling and further studies should be made.

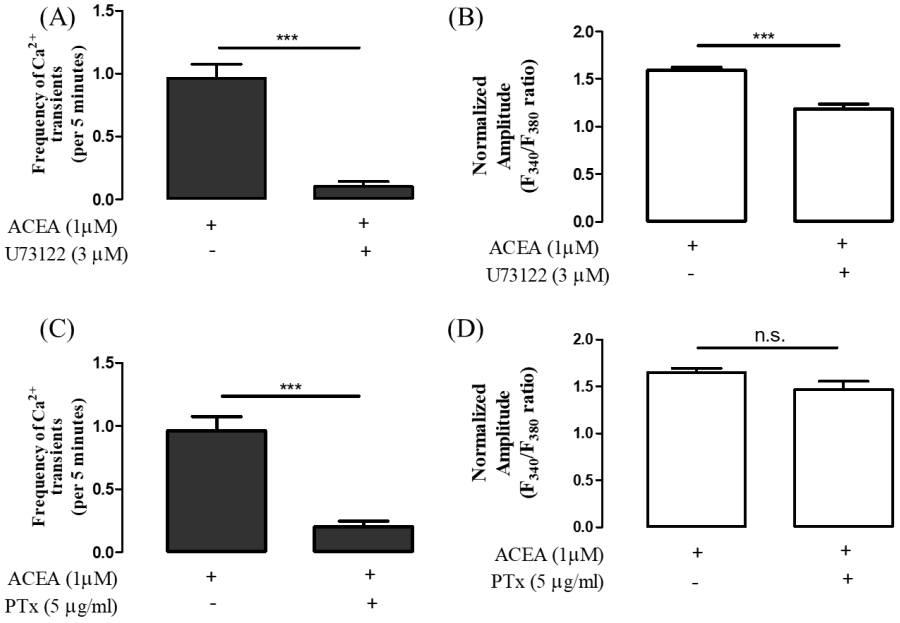

Figure 4.6-Influence of PLC and $G_{i/o}$ coupled protein in CB1R calcium signalling.
(A,B) Treatment with U73122(3 µM) 15 minutes prior to ACEA incubation for 20 minutes of experiment. (C,D) Treatment with Pertussis Toxin (5 µg/mL) 15 minutes prior to application of ACEA for 20 minutes. Data are expressed as Mean ±S.E.M from 21-49 responsive cells from 3-7 independent cultures.; ***$P < 0.001$ assessed by Student's *t*-test.

4.3. CB1R effect in astrocytic glutamate transport

4.3.1. Glutamate transport in primary culture of astrocytes

Before testing CB1R activation effect on glutamate uptake, the kinetic constants of each glutamate transporter was determined. According to the literature, in astrocytes, the main glutamate transporters are GLAST and GLT-1, being the ones chosen to study in this work. To do so, glutamate uptake assays were made in the presence of WAY-213613 (10 µM) and UCPH-101 (10 µM), which are selective antagonists of GLT-1 and GLAST, respectively. Firstly, a time course assay was performed to see for how long the glutamate transport was linear. For that, astrocytes were incubated with 100 µM of glutamate for 1, 2, 3, 4, 6, 8, 10, and 14 minutes (Appendix 1 - Figure 7.1). It was observed that transport remained linear for approximately 4 minutes (*n*=3, 3 replicates per condition), being this the time selected for all the posterior experiments. In the presence of GLT-1 antagonist, glutamate transport had a substantial variability and it was much lower than in the presence of UCPH-101, the GLAST antagonist. Due to this, it was opted to only study GLAST activity from this moment on. In fact, GLT-1 expression in astrocytes is low when there are no soluble factors released from neurons[104]. Next, a Michaelis-Menten curve was obtained, and, in order to determinate the K_m and V_{max} values of GLAST transporter, astrocytes were incubated with a glutamate transport buffer containing 10, 30, 60, 90, 120, 200, 400, and 1000 µM of glutamate for 4 minutes. In cultured astrocytes, GLAST was responsible for about 70% of the transport which was why we only worked with this transporter for all our posterior experiments. In control conditions (without addition of ACEA and/or other drugs except for GLAST antagonist [UCPH-101]), GLAST transport was shown to have a K_m of about 234 ± 47.18 µM and a V_{max} of approximately 1304 ± 110.4 pmol/mg protein/minute (Figure 4.7, Table 1).

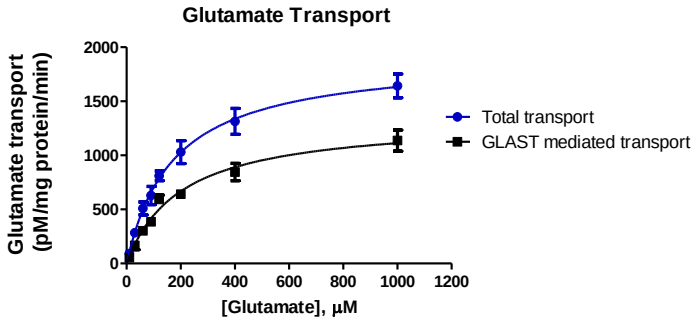

Figure 4.7- Glutamate transport in cortical astrocytes.
Before studying CB1R effect in glutamate, a saturation curve was made with increasing glutamate concentrations(10, 30, 60, 90, 120, 200, 400 and 1000 µM) for 4 minutes(time when transport was linear). Counts per minute (CPM) was normalized to amount of protein in each replicate. Data are expressed as Mean ± S.E.M. of 3 independent cultures, 3-4 replicates per *n*.

4.3.2. CB1R activation potentiates GLAST activity

Since calcium signalling has been implicated in neurotransmitter uptake in astrocytes[97,101], and it was observed in the present work that CB1R activation led to calcium transients in cultured cortical astrocytes, it was next questioned if the calcium signalling triggered by CB1Rs in astrocytes could modulate glutamate uptake mediated by GLAST transporter. Glutamate uptake is crucial for the maintenance of homeostasis of the CNS. CB1Rs effect in glutamate receptors like NMDAR is well described[39,105,106] but, to our knowledge, no studies have been made on the effect of CB1R or any other type of cannabinoid receptor upon glutamate uptake. To evaluate the CB1R effect on astrocytic glutamate transport and to maintain the experimental conditions performed in the experiments of *Calcium Imaging*, astrocytes were treated in a similar way to calcium signalling experiments with ACEA and the other tested drugs before the glutamate uptake assays. Firstly, astrocytes were incubated with 1 µM of ACEA for 20 minutes and a saturation curve with increasing concentrations of glutamate was performed to study if the synthetic cannabinoid has an effect in the kinetic constants K_m and V_{max}. Changes in V_{max} imply a difference in the number of transporter binding sites, which are correlated with translocation of the transporter from the plasma membrane and changes in K_m are indicative of changes in the affinity of individual transporters. For reasons mentioned before, only GLAST activity was assessed. A Michaelis-Menten fitting of saturation curve for GLAST-mediated glutamate transport in the presence of ACEA (1 µM) for 20 minutes revealed a significant increase of V_{max} (Control: 1304 ± 110.4 and ACEA: 1880±191, Table 1, $P < 0.05$), with no significant change in the constant K_m. An increase in this parameter highly suggests that the astrocyte treatment with ACEA increases the number of functional GLAST transporters in the astrocytic plasma membrane. When incubated with AM251, 15 minutes prior to the ACEA incubation, the ACEA effect was completely lost (Figure 4.8 B, n=3 $P < 0.05$). Taken together, these results highly suggest that ACEA potentiates glutamate uptake by increasing the number of functional glutamate transporters GLAST by activation of CB1R in astrocytes.

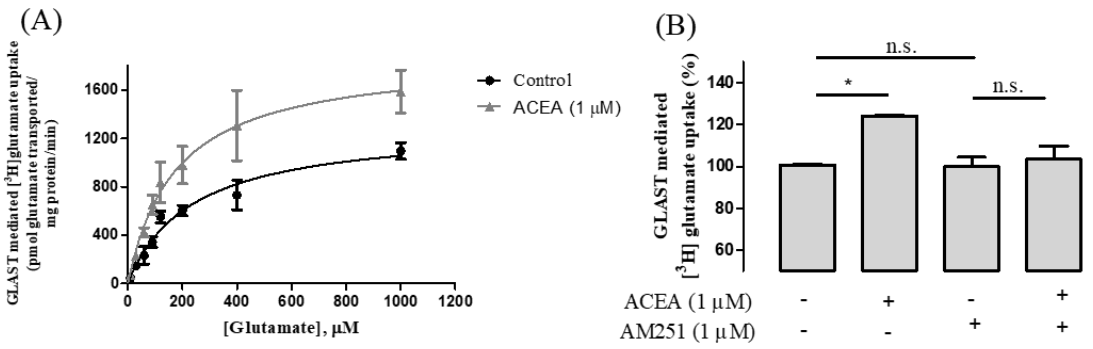

Figure 4.8 CB1R effect on GLAST glutamate transport.
(A) Saturation curve, depicting the amount of glutamate taken as function of the concentration of glutamate in the absence (black squares) or presence of ACEA, 1 µM (grey triangles). (B) Prior incubation of CB1R antagonist AM251(1 µM) ceases CB1R modulation on glutamate transport. The ordinates represent GLAST mediated [^{3}H] glutamate uptake as percentage of the control value in the same experiment, which was taken as 100%. Data are expressed as Mean ± S.E.M. of 3 independent cultures;*$P < 0.05$ assessed by one-way A-NOVA followed by Bonferrini correction.

Table 1. Kinetic Constants (K_m and V_{max}) of glutamate transport mediated by GLAST.
Data are expressed as Mean ± S.E.M. of 3 independent cultures; *$P < 0.05$ assessed by Student t-test.

GLAST	K_m	V_{max} (pmol/mg protein/min)
Control	234.6 ± 47.18	1304 ± 110.4
ACEA	177.2 ± 45.80	1880 ± 191.0*

4.3.3. CB1R-mediated GLAST potentiation is PLC but PKC independent

In this work and others[39], it is strongly suggested that CB1Rs in astrocytes are $G_{q/11}$ coupled since their posterior action is ceased in the presence of a PLC antagonist, a downstream protein of the $G_{q/11}$ pathway. Protein kinase C (PKC) is a downstream protein of PLC so it is also a possible candidate in the pathway. To further inspect, the CB1R-mediated potentiation of GLAST activity, we assessed the role of $G_{q/11}$ downstream proteins. Prior to the cannabinoid incubation, PLC and PKC activity were inhibited by incubating the primary astrocytes with U73122 (3 µM) and GF 109203X (1 µM), respectively, 15 minutes prior to ACEA incubation. PLC inhibition abolished the ACEA effect (Figure 4.9 A). Interestingly, incubation of GF 109203X alone led to an increase of GLAST activity, similar to when astrocytes were incubated with ACEA alone (ACEA: 123 ± 2.28% vs GF 109203X: 119 ± 0.0724%, Figure 4.9 B). This effect was not additive when astrocytes were incubated with ACEA and GF 109293X together. These results suggest that PLC is required for the CB1R effect in astrocytic glutamate effect and that PKC tonically inhibits GLAST activity and appears to have no role in the metabotropic receptor CB1R studied in this work, although further studies need to be done.

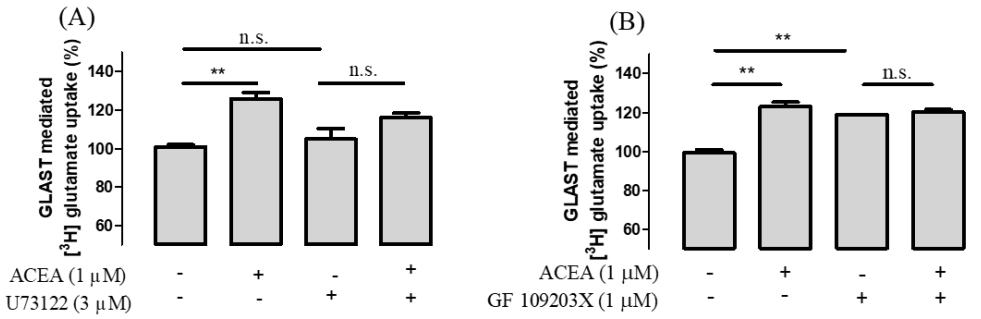

Figure 4.9- CB1Rs modulate GLAST activity in cortical astrocytes, through a PLC-dependent mechanism.
Application for 20 minutes of ACEA (1 µM) does not increase GLAST transport activity in U73122 treated cells. The ordinates represent GLAST mediated [^{3}H] glutamate uptake as percentage of the control value in the same experiment which was taken as 100%. Data are expressed as Mean ± S.E.M.;** $P < 0.01$; assessed by one-way A-NOVA followed by Bonferrini correction.

4.3.4. CB1R-mediated GLAST potentiation is calcium signalling dependent

So far, it was showed that ACEA in cultured cortical astrocytes led to calcium transients and a potentiation in GLAST activity. Both consequences appear to be mediated by CB1R and to be PLC-dependent. Some studies state that some astrocytic mediated-calcium signalling led to the increase of neurotransmitter transport such as GABA or glutamate[97,101,107]. For this reason, we wanted to verify if the CB1R-mediated calcium transients led to the potentiation of GLAST activity or if both consequences were parallel to each other. To do so, before glutamate uptake, cells were incubated with BAPTA-AM, a permeable calcium specific chelator that ceases calcium transients. When incubated with this chelator and the synthetic cannabinoid, there was a significant GLAST activity decrease when compared to astrocytes incubated with ACEA alone (Figure 4.10, $n = 3$ independent cultures, 3-4 replicates per n). This led us to believe that CB1R calcium transients and glutamate increase are not parallel consequences of CB1R activation but in fact CB1R-GLAST potentiation in astrocytes is mediated by the calcium transients that are also triggered by CB1R activation.

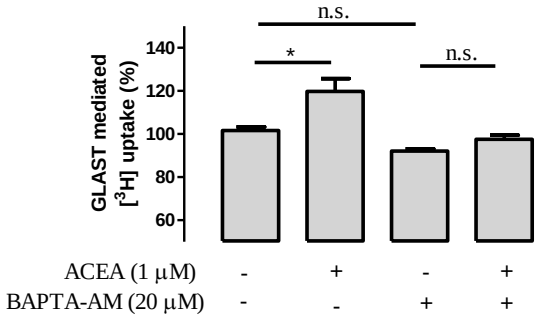

Figure 4.10-Influence of calcium signalling on GLAST-mediated glutamate transport.
Exogenous application of ACEA (1 µM) for 20 minutes does not potentiate GLAST activity transport activity in BAPTA-AM-loaded cells (20 µM for 30 minutes). The ordinates represent GLAST mediated [^{3}H] glutamate uptake as percentage of the control value in the same experiment which was taken as 100%. Data are expressed as Mean ± S.E.M.; *$P < 0.05$ from 3 independent cultures; assessed by one-way A-NOVA followed by Bonferrini correction.

4.4. Modulation of CB1R-mediated calcium signaling by adenosine receptors

4.4.1. Astrocytic A_{2A} receptor activation attenuates CB1R mediated calcium signalling

Because adenosine $A_{2A}R$ are coupled to G_s proteins, these receptors are not usually directly implicated in calcium signalling. However, some reports have implicated PKA in calcium signalling[108,109], being

PKA a downstream protein of the G_s signalling pathway. There are also numerous reports of interactions between adenosine $A_{2A}R$ and CB1R, but none of them in glial cells[26,110]. To study the adenosine $A_{2A}R$ effect upon CB1R-mediated calcium signalling, astrocytes were incubated with a selective adenosine $A_{2A}R$ agonist, CGS21680 (30 nM). When adenosine $A_{2A}R$ were activated with CGS21680 (30 nM), before CB1R activation, the calcium transients frequency (ACEA: 0.964 ± 0.109 transients per 5 minutes and ACEA+CGS21680: 0.227 ± 0.081 transients per 5 minutes, Figure 4.10 A, $P < 0.001$) was significantly decreased in comparison to ACEA alone, leading to the conclusion that adenosine $A_{2A}R$ activity attenuates CB1R-mediated calcium signalling. The CGS21680 by itself elicited some transients (CGS21680: 0.344 ± 0.077 transients per 5 minutes) suggesting the participation of adenosine receptors in astrocytic calcium signalling. The difference of frequency of these transients was not significant when compared with CGS21680 and ACEA incubation together (ACEA+CGS21680: 0.227 ± 0.081 transients per 5 minutes and CGS21680: 0.344 ± 0.077 transients per 5 minutes), suggesting that the occurrence of the transients were elicited by adenosine receptor only. As for the amplitude, a similar behavior was observed where CGS21680 and ACEA together had lower amplitude when compared to ACEA alone (ACEA: 1.59± 0.03 and ACEA+CGS21680: 1.43 ± 0.05, Figure 4.10 B, $P < 0.05$) and no significant difference between the amplitude of CGS21680 alone and ACEA+CGS21680. Next, it was evaluated the effect of adenosine $A_{2A}R$ blockade, with a selective adenosine $A_{2A}R$ antagonist, SCH58261, upon CB1R-mediated calcium signalling. When comparing the occurrence of transients between SCH58261 alone and SCH58261 together with ACEA, the difference of frequency was significant (ACEA: 0.964 ± 0.109 and ACEA + SCH58261:1.49 ± 0.25, $P < 0.001$, Figure 4.11 B), suggesting that an increase of transients occurred when there is a blockade of adenosine $A_{2A}R$ and an activation of CB1R. Interestingly, ACEA together with SCH58261 showed a lower amplitude compared with ACEA alone (ACEA: 1.59±0.03 and ACEA+SCH58261: 1.46 ± 0.016, Figure 4.11 D, $P < 0.001$). It was also observed a significant difference between SCH58261 and SCH58261 together with ACEA (Figure 4.11 D, $P < 0.001$) suggesting blockade of $A_{2A}R$ changes CB1R-mediated Ca^{2+} transients frequency and amplitude.

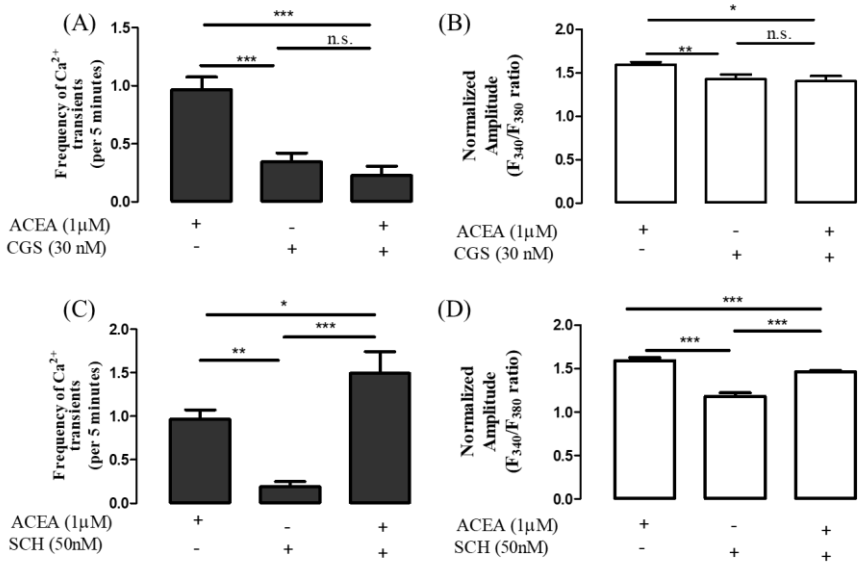

Figure 4.11-Adenosine $A_{2A}R$ effect on CB1R-mediated calcium transients.
(A,B) Astrocytic Adenosine $A_{2A}R$ was previously activated, (by application of CGS21680, 30 nM) or (B,C) inhibited (by incubation of SCH) before CB1R activation. Data are expressed as Mean ±S.E.M from 32-49 cells from 3-7 independent cultures; ***P<0.001, **P < 0.01,*P < 0.05; accessed by one-way ANOVA followed by Bonferroni correction.

One consequence of adenosine $A_{2A}R$ activation is the PKA activation mediated by cAMP accumulation. Due to previous results, it is suggested an effect of PKA in calcium signalling. Our preliminary data

suggests that PKA inhibition with cAMP-Rp (100 µM) elicits calcium transients. Posterior ACEA incubation appears to cease the PKA inhibition effect by stopping calcium transients (Appendix 3-Figure 7.1 K). However, these transients were not considered for the statistical analysis since the potential peaks were not higher or the same as the mean plus 15 times the S.E.M.. While this result suggests that PKA tonically inhibits calcium transients, further experiments must be performed to validate this observation.

4.4.2. Astrocytic $A_{2A}R$ activation effect upon glutamate uptake

Since CB1R effect on glutamate transport depended on the calcium signalling generated by the same receptor, it was questioned if $A_{2A}R$ effect of CB1Rs calcium signalling would also imply some effect on glutamate transport. To evaluate this hypothesis, the effect of CB1R activation upon GLAST activity in astrocytes was accessed in presence of specific agonists and antagonists of $A_{2A}R$. Inhibition of adenosine $A_{2A}R$ with SCH58261 (50 nM) annulled CB1R-mediated glutamate uptake increase (Figure 4.12 A). SCH58261 (50 nM) by itself had no effect upon GLAST transporter (Figure 4.12 A). In the presence of CGS21680 (30 nM) alone, there was a significant increase of glutamate uptake mediated by GLAST of 29.4 ± 4.3 %. When CGS21680 (30 nM) was tested together ACEA (1 µM), it was observed a potentiation of GLAST activity similar to the one obtained for CGS21680 (30 nM) alone. These results are the opposite of the results obtained for calcium assays where $A_{2A}R$ activation constrains CB1R-mediated calcium transients frequency and its inhibition increases CB1R-calcium signalling. Not only that, it is reported in other studies that activation of adenosine $A_{2A}R$ decreases uptake in astrocytes[86,111]. These opposite effects may be related with the fact that, for glutamate uptake assays, cells are in a much higher concentration that in calcium imaging assays, so the endogenous adenosine can have an impact in the astrocytes, and that will be related with an effect of the selective $A_{2A}R$ antagonist. Presence of adenosine can also activate adenosine A_1R which has been showed to interact with adenosine $A_{2A}R$[85]. Further studies have to be made in the presence of adenosine deaminase (ADA).

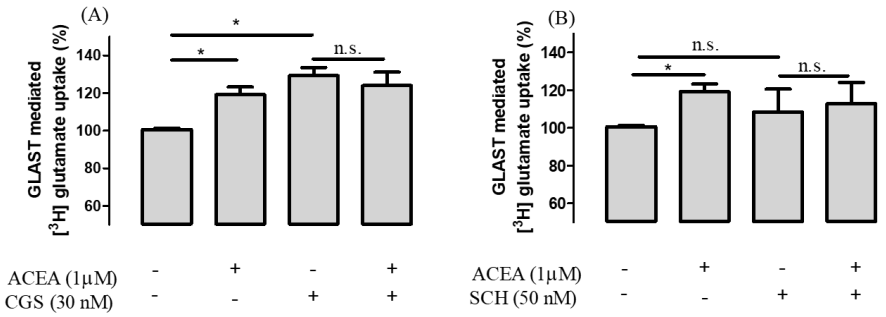

Figure 4.12 – Adenosine $A_{2A}R$ modulation on CB1R potentiation in glutamate uptake.
Previous application of adenosine $A_{2A}R$ agonist and antagonist before exogenous application of ACEA (1 µM) for 20 minutes did not alter GLAST mediated glutamate uptake. The ordinates represent GLAST mediated [^{3}H] glutamate uptake as percentage of the control value in the same experiment which was taken as 100%. Data are expressed as Mean ± S.E.M.; $P < 0.05$ from 2 independent cultures; assessed by one-way A-NOVA followed by Bonferrini correction.; *$P < 0.05$

4.4.3. Astrocytic adenosine A1 receptor inhibition effect in CB1R-mediated calcium signalling

Besides adenosine $A_{2A}R$, A_1 receptors are also present in astrocytes and are implicated in many processes such as GABA uptake, calcium flux, and glutamate and dopamine signaling[86,96]. To observe the possible impact of adenosine A_1 receptor on CB1R-mediated calcium signalling, astrocytes were pre-incubated with adenosine A_1 receptor antagonist DPCPX (50 nM). In this condition, ACEA incubation still elicited calcium transients even though the frequency was decreased (ACEA: 0.964 ±

0.109 transients per 5 minutes and ACEA+DPCPX: 0.344 ± 0.077 transients per 5 minutes). Unlike SCH58261, DPCPX alone did not elicit calcium transients. Similar to PTx, amplitude of transients elicited by CB1R did not differ when astrocytes were previously incubated with DPCPX (Figure 4.13 B). However it is important to keep in mind that this is n = 1 with 16 responsive cells and further studies need to be made.

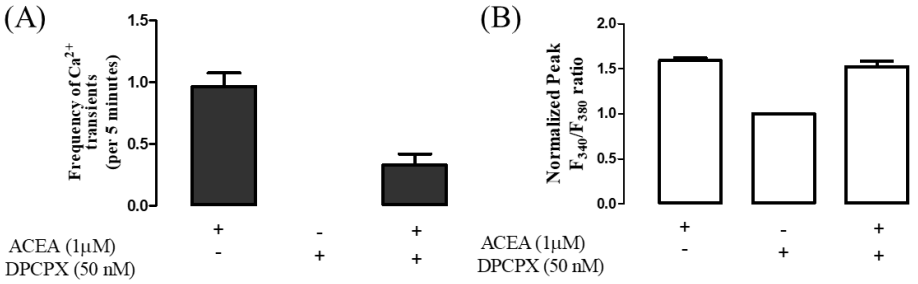

Figure 4.13 Adenosine A_1 receptor modulation on CB1R-mediated calcium transients.
Previous application of adenosine A_1 receptor antagonist before exogenous application of ACEA(1 μM) for 20 minutes cease calcium transients. Data are expressed as Mean ± S.E.M from 16 responsive cells from 1 independent culture.

5. Discussion

The main finding of the present work is that CB1R activation triggers calcium transients in astrocytes through a mechanism that involves the PLC activation and mainly the intracellular calcium stores. The CB1R activation, through the calcium signalling, enhances glutamate GLAST transport activity probably by increasing the number of functional GLAST transporters in the plasma membrane. It was also observed a crosstalk between adenosine receptors and CB1R, with implications for calcium signalling and glutamate uptake.

Astrocytes are known to perform crucial tasks in the CNS regarding numerous things such as synaptic transmission, inflammation, ionic environment, among others. These cells are able to receive and transmit information and also communicate with each other through calcium signals. Calcium signalling in astrocytes has been related to numerous astrocytic tasks like neurotransmitter uptake and gliotransmitter release, making astrocytes a relevant player in the synapse[61]. Cannabinoid system is a modulatory system with tremendous impact in the CNS being implicated not only in behavior but also in learning and synaptic transmission[53]. In these glial cells, the endocannabinoid receptor CB1R activation triggers intracellular calcium elevations, which then triggers gliotransmitters release[39]. So far no correlations between glutamate uptake and CB1Rs have been made. On the other hand, since neurotransmitter uptake has been correlated with calcium signalling, CB1R in astrocytes could be a potential modulator of neurotransmitter uptake. Thus, the aim of this work was to evaluated the influence of CB1R upon glutamate uptake and calcium signalling and to verify if these two consequences were related.

To start, expression of CB1R and main glutamate transporters GLT-1 and GLAST transporters in cortical astrocytes was evaluated. To this end, immunocytochemistry assays were performed. Both receptor and transporters were found to be expressed in cultured cortical astrocytes. GLAST and GLT-1 expression was not uniform. GLT-1 seemed to have an higher expression in the area surrounding the nucleus and GLAST seemed to have an higher expression in the membrane and intracellular space. This difference could be explained by the fact that astrocytic GLT-1 expression depends of neuronal factors. In an earlier study, it was found that cultures of astrocytes in the absence of neurons express GLAST but very little GLT-1[112] and that co-culture of astrocytes and neurons increases GLT-1 and GLAST

expression[113]. This could explain the differences observed in glutamate uptake where GLAST in cultured astrocytes was revealed to be the main transporter. Probably there are mechanisms that can activate the translocation of GLT-1 to the plasma membrane under specific conditions. For instance, it would be interesting to evaluate if, for example, the excitotoxicity with glutamate changes the immunostaining obtained for the glutamate transporters.

In a similar way to what was already described in literature[39], activation of CB1R in astrocytes triggered calcium transients. In this work in particular, it was observed that both frequency and amplitude were dose-dependent of the CB1R agonist. These transients were PLC and intracellular calcium transient dependent, highly suggesting that astrocytic CB1R is coupled to $G_{q/11}$, since both these characteristics agree with the activation of these type of proteins. Low extracellular calcium did not cease the calcium transients but had an impact decreasing both frequency and amplitude of these. This suggests that a membrane ion channel plays a role in these transients mediated by CB1R activation. One potential candidate is a type of transient receptor potential cation channel (TRPC). These ion channels are activated by diacylglycerol (DAG), a molecular second messenger that is downstream of PLC, which was proved in this work that is activated by CB1R and expressed in astroglia but also to modulate their calcium signalling[114]. Therefore, it can be theorized that CB1R-dependent PLC activation leads to accumulation of DAG which then activates TRPC triggering a calcium influx from the extracellular space to the cytosol. In fact, another subtype of TRP channel, the TRPV4 is expressed in a subset of cortical astrocytes and can bind to endocannabinoids[115], also being a potential candidate. However, without further studies this cannot be confirmed, and other ion channels must be kept in question like the sodium-calcium exchanger (NCX), an important calcium channels.

Interestingly, PTx also had an impact in these calcium transients. When the protein $G_{i/o}$ was inhibited with PTx, an increase of basal calcium level was observed together with the occurrence of calcium transients. This does not necessarily mean that astrocytic CB1R is coupled to $G_{i/o}$. $G_{i/o}$ downstream proteins like AC or PKA activity can have an impact on astrocytic CB1R or on calcium signalling directly. Some studies report PKA activation phosphorylates IP3R1 and IP3R2 increasing the probability of the coupled ion channels to open in the presence of IP3 (like activating $G_{q/11}$ coupled receptors)[108]. In another study[29], where CB1R was in fact proved to be coupled to an $G_{q/11}$ protein, a previous inhibition of $G_{i/o}$ protein increased CB1R-mediated calcium transients amplitude. IP3R2 have been nominated the putative functional IP3R in glial cells[116]. However, in IP3R2KO animals, it had no impact in calcium signalling triggered by mGluR1 in astrocytes[117]. Beyond that, an IP3R2/IP3R3KO ceased to have calcium signalling in the same conditions. This could reveal that CB1R activation triggers not just only IP3R2 but also IP3R3. Interestingly, the PKA activation is described to cease IP3-medited calcium signalling[114]. This highly suggests that cAMP and/or PKA can modulate the different types of IP3 receptors in distinct ways. However, further studies must be performed.

After establishing the mechanisms behind CB1R-mediated calcium signalling in primary culture of astrocytes, the focus of this work was moved to the study of CB1R activation upon glutamate transporters. It was found that astrocytic CB1R activation potentiated glutamate uptake, the effect being shown in an increase of the kinetic constant V_{max} in GLAST activity with no difference in K_m. Hence, activation of CB1R increases glutamate uptake by possibly increasing the number of transporter binding sites at the plasma membranes. In other words, it increases the number of functional transporters in the astrocytic plasma membrane without changing the affinity of these sites. It would be interesting to evaluate in future work the level of GLAST transporters at membrane level after activation of CB1R. Other important issue is the occurrence of constitutive recycling of neurotransmitter transporters in neurons and astrocytes[118,119]. Therefore, if this would apply to GLAST transporter in astrocytes,

enhanced expression of GLAST on astrocytic surface membranes could result either from inhibition of endocytosis or from enhancement of its recycling back to the membrane.

Similarly to calcium transients, GLAST activity was also PLC dependent. Some reports have been made that state that calcium signalling modulates neurotransmitter uptake, more specifically glutamate transport[97]. Having this in mind, the next step was to observe if the calcium transients and potentiation of GLAST activity were parallel consequences of astrocytic CB1R activation or if they were sequential. More specifically, if calcium transients led to the enhancement of GLAST activity. In the presence of a calcium chelator, BAPTA-AM, ACEA lost its effect in GLAST activity. These results conclude that CB1R effect on glutamate uptake is dependent on calcium signalling generated by the same receptor.

One unanswered question in this work is how the calcium transients triggered GLAST activity potentiation. PKC is a downstream protein of PLC and therefore calcium signalling, and its activity has been correlated with GLAST activity. Curiously, the PLC's downstream protein PKC did not seem to have an active role in CB1R modulation. In fact, inhibition of PKC alone increased GLAST activity, suggesting PKC has an intrinsic effect on GLAST-mediated transport. This is consistent with previous reports where activation of PKC decreased GLAST activity[4]. This suggests that PKC tonically inhibits glutamate transport and therefore it is not implied in the CB1R modulation. One possible pathway in how calcium signalling potentiates GLAST activity is trough activation of Calcium-Calmodulin-Dependent Protein Kinase II (CaMKII). This kinase is regulated by the Calcium/calmodulin complex. This protein is a calcium downstream sensor[102] and its inhibition decreases GLAST glutamate transport activity[120]. However, it is important to keep in mind that different subtypes of CaMKII exist and their ability to decode calcium signalling differs. Nevertheless it is important to refer that, to our knowledge, this is the first time that glutamate uptake modulation by CB1R in any type of brain cell is described. Due to the observed effect of $G_{i/o}$ inhibition on CB1R effect on calcium, we postulated that some $G_{i/o}$ or G_s coupled receptors could have some effect on CB1R in astrocytes. Adenosine receptors, $A_{1A}R$ and $A_{2A}R$, are $G_{i/o}$ and G_s coupled proteins, respectively. Since these receptors are expressed in astrocytes, the next step was to evaluate the possible effect of $A_{2A}R$ in astrocytic CB1R. Activation of adenosine $A_{2A}R$ attenuated calcium transients triggered by CB1R. A more interesting result was when adenosine $A_{2A}R$ was inhibited the CB1R-mediated calcium transients were amplified. These results suggest adenosine $A_{2A}R$ tonically modulate astrocytic CB1R calcium signalling, showing a novel interaction between these two proteins in astrocytes. This is not the first time an interaction between these two receptors has been observed. Many studies have either showed interaction and/or dimerization in neurons. Pre-synaptic adenosine $A_{2A}Rs$ diminish CB1R effect in glutamatergic transmission[92] and the majority of glutamatergic terminals that express adenosine $A_{2A}R$ also co-express CB1Rs implying that in most cases their activity depends on adenosine $A_{2A}Rs$. Most corticostriatal glutamatergic terminals expressing $A_{2A}R$ also co-express CB1R and it has been suggested that their pharmacological interactions depend on their heteromerization. When adenosine A_1R were inhibited, no calcium transients were recorded. In this condition, when CB1R was activated calcium transients still occurred although their frequency seemed to be decreased, with no apparent changes in amplitude. This behavior was similar to when $G_{i/o}$ coupled protein was inhibited which suggest some sort of correlation.

Interestingly, activation of adenosine $A_{2A}R$ appeared to increase astrocytic glutamate uptake independently of CB1R activation. This is goes against other works where activation of these receptors lead to a decrease of glutamate uptake[86,96]. However, it is important to keep in mind that in the experiments present in this work, no endogenous adenosine was removed. Endogenous adenosine can bind to A_1R and $A_{2A}R$ among other adenosine receptors, activating them, which can influence the obtained results and its presence has been showed to interfere with astrocytic GABA uptake[85,121]. Further studies have to be performed when endogenous adenosine needs to be removed with ADA, for

instance, in order to understand the impact of adenosine $A_{2A}R$ in CB1R-mediated increase in glutamate uptake. In fact, there are studies stating modulation of adenosine receptors in neurotransmitter uptake in astrocytes[85,86]. To our knowledge, this is the first report of an interaction between these two receptors in glial cells.

Accumulation of extracellular glutamate leads to toxicity in the brain. Decreased glutamate uptake has been reported in many neurodegenerative diseases like PD, among others. In fact, the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) decreases glutamate uptake in astrocytes[122]. This is particularly interesting since CB1R activation has the exact opposite effect. It would be interesting to further inspect these two components in the context of disease and possibly having a pharmacological approach to elicit CB1Rs to increase glutamate transport. At the same time, disruption in the CB1R expression has also been associated with neurodegenerative diseases where deficient glutamate transport is also implicated.

In summary, a model of CB1R activation in brain astrocytes is proposed in Figure 5.1. In this model it is proposed that CB1R activation leads to PLC activation that leads to calcium transients mediated by IP3R. These Ca^{2+} transients then increase GLAST activity probably by increasing the number of functional GLAST transporters in the membrane. This increase is modulated by $A_{2A}R$ and activation of this receptor inhibits CB1R effect.

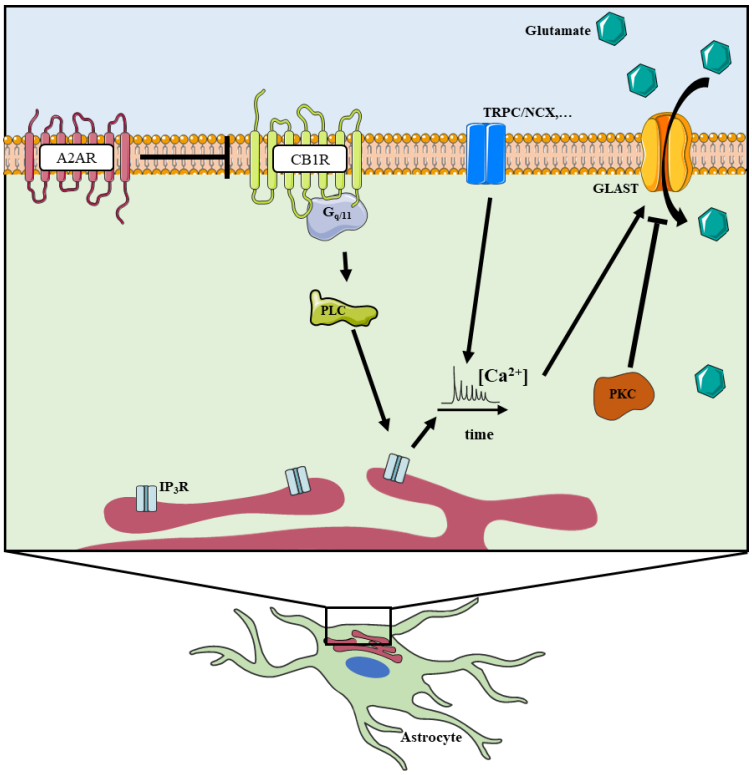

Figure 5.1-CB1R activation model in cortical astrocytes.
CB1R activation elicits calcium transients in $G_{q/11}$-PLC-Insp3-dependent manner. This phenomenon phosphorylates CaMKII which increases GLAST activity by increasing the number of functional transporters in the membrane, probably by trafficking from internal vesicles. Adenosine $A_{2A}R$ modulate intrinsically CB1R activity by attenuating its activity.

6. Conclusions and Future perspectives

The presented work demonstrates a novel modulation of glutamate uptake by CB1Rs. It also represents a novel interaction between adenosine $A_{2A}R$ and CB1Rs in astrocytes. More specifically it shows that in cortical cultured astrocytes:

- CB1Rs are expressed and functional in cortical astrocytes;
- CB1R activation triggers calcium transients via PLC;
- Intracellular calcium stores are essential for occurrence of calcium transients mediated by CB1R, but extracellular calcium also has a significant effect;
- CB1R potentiates glutamate transport by increasing number of functional transporters GLAST in the membrane;
- $A_{2A}R$ tonically inhibits CB1R calcium signalling;

In the future, additional experiments/questions to better understand the mechanism beyond the results here presented should be explored to answer if the interaction between these two receptors is bidirectional and if the impact of CB1R elicited glutamate uptake potentiation is relevant in the context of synaptic transmission and/or mental diseases.

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Appendix

Appendix 1- Time course curve for Glutamate transport

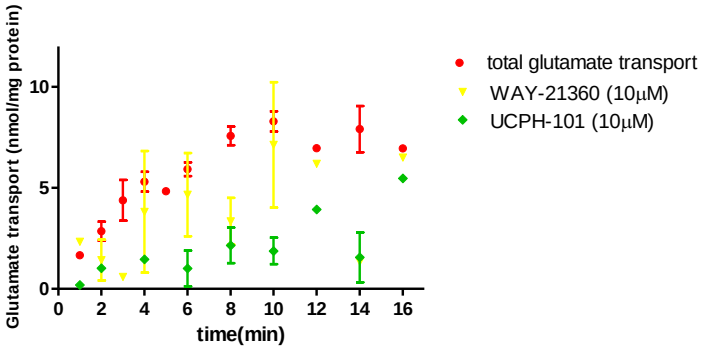

Figure 7.1-Time course of glutamate transport.
To observe the effect of GLAST and GLT-1, glutamate uptake assays were made in the presence of WAY-21360(GLT-1 antagonist) and UCPH-101(GLAST antagonist). Glutamate transport was linear for 4 minutes in the 3 conditions studied.

Appendix 2 - Matlab script for Calcium Imaging statistical Analysis

```
clear all
close all
file='ml-acea.xlsx';
marge=15;
fc=0;
compensacao=300;
umaauma=1;
baseline=300;  % 5 minutos
ni=1200;
nl=2400;
duracao_min=2;   % acima da margem com duração maior que
duracao_max=300000;   % abaixo da margem com duração maior que
%mav=1;

calcio=xlsread(file);
t=calcio(:,1);
calcio(:,1)=[];
[maxi, kf]=max(t>baseline);   % indice do array funçao do tempo
[maxi, nii]=max(t>ni);
[maxi, nll]=max(t>nl);
[maxi, comp]=max(t>compensacao);
nduracao_min=round(duracao_min/5,0);   % número de amostra do pico
nduracao_max=round(duracao_max/5,0);   % número de amostra do pico
[li, cl]=size(calcio);

kf=kf-1;
for i=1:cl  % célula a célula
   m=(calcio(comp,i)-calcio(1,i))/(comp-1);  % declive da compensação
   declives(i)=m;
   d=calcio(:,i)-fc*m*(1:li)';            % compensação

   medcel(1,i)=mean(d(1:kf));
   medcel(2,i)=std(d(1:kf));
   medcel(3,i)=medcel(1,i)+marge*medcel(2,i);

   treshold=medcel(3, i);
   plot(t,d);
```

```
    hold on
    x(1:li)=treshold;
    plot(t,x,'r');

    pico=d>treshold;
    pico(1:nii-1)=0;
    pico(nll:end)=0;
    plot(t,1.3*treshold*pico)
    plot([ni ni], [0 max(d)], 'k')
    plot([nl nl], [0 max(d)], 'k')
    axis tight
    hold off
    ptr=nii;
    j=2;
    while(ptr<nll)
        [maxi, niii]=max(pico(ptr:nll));
        niii=niii+ptr-1;
        [mini, nlll]=min(pico(niii:nll));
        nlll=nlll+niii-2;
        if(nlll<niii)
            if(j==2)
                amp(:,i)=0;
                fprintf('Warning: Célula %d vazia!!!!!!', i);
            end
            break;
        end
%       if( (nlll-niii+1>nduracao_min) && (nlll-niii+1<nduracao_max) && (nii~=niii) )
        if( (nlll-niii+1>nduracao_min) && (nlll-niii+1<nduracao_max)) % && (nii~=niii) )
            [amp(j,i), ind]=max(calcio(niii: nlll,i));
            j=j+1;
        end
        ptr=nlll+1;
        amp(1,i)=j-2;
    end
    if(umaauma)
        fprintf('Célula %d\n', i);
        disp(amp(:,i))
        jj=input('Célula seguinte...', 's');
    end
end
medcel
amp
x=size(amp);
surfc(1:x(2), 1:x(1), amp, 'Edgecolor', 'none');
axis xy; axis tight; colormap(jet); view(0,90);

surf(1:cl, t, calcio)
axis xy; axis tight; colormap(jet); %view(0,90);
figure
plot(calcio)
axis tight

s=input('Quer guardar?','s')
if(s~='n')
    amp(1, cl+1)=marge;
    amp(2, cl+1)=fc;
    amp(3, cl+1)=300;
    amp(4, cl+1)=duracao_min;
    amp(5, cl+1)=duracao_max;
```

```
    s=[s '.xlsx'];
    xlswrite(s, amp)
end
```

Appendix 3 – Calcium Imaging Representative Curves

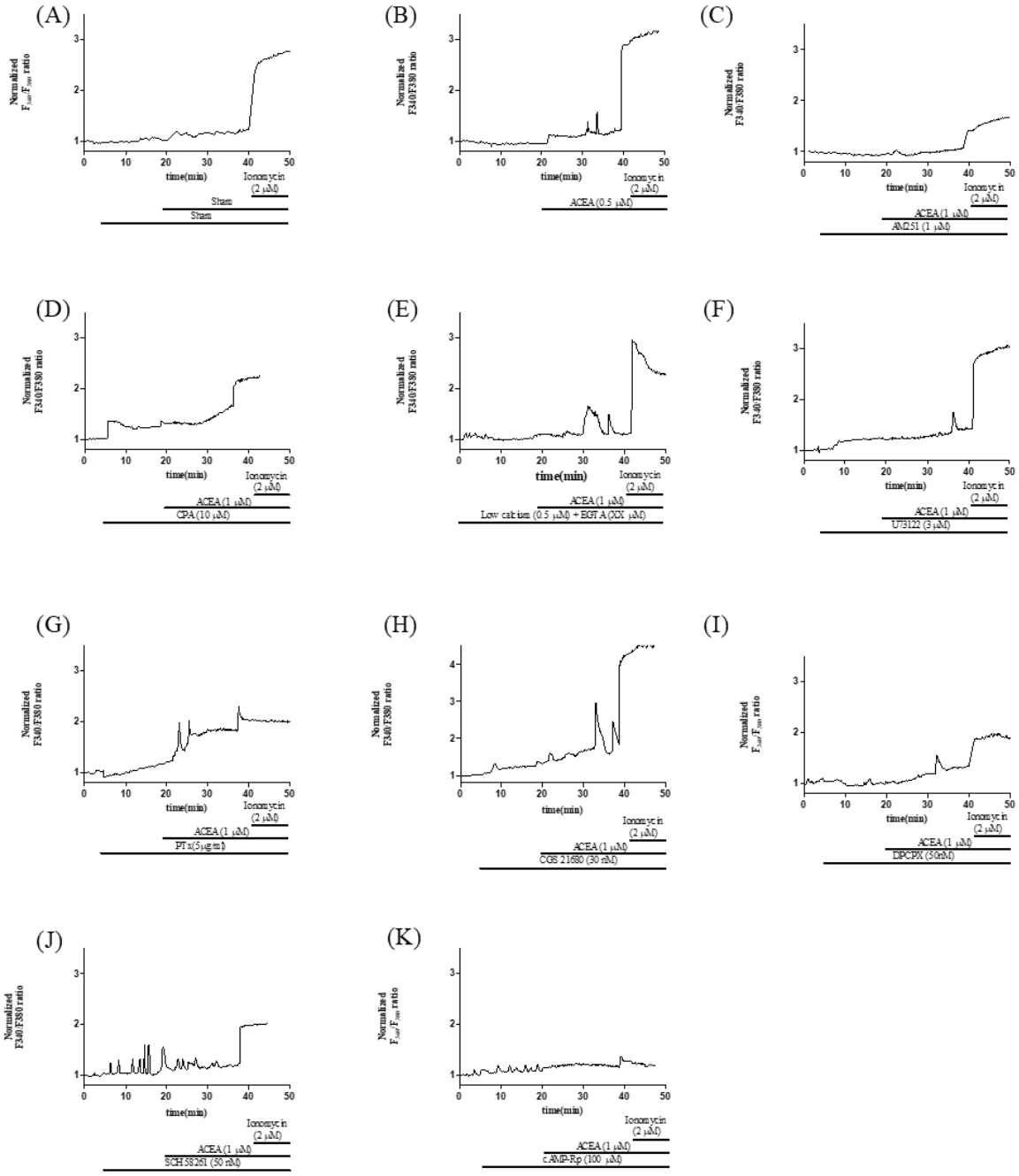

Figure 7.2-Representative curves of CB1R activation upon calcium transients in cultured astrocytes.
Each condition is indicated on the bottom of each image.